



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent Application of

Applicant: Cindy ORSER et al.

Application No.: 10/728,246

Filing Date: 4 December 2003

Confirmation No.: 8108

Title: DETECTION OF CONFORMATIONALLY ALTERED PROTEINS AND PRIONS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION TO MAKE SPECIAL

Sir:

Applicants request that this application be accorded Special Status on the basis of the age of inventor Eugene A. DAVIDSON.

As stated on the attached Statement of inventor DAVIDSON, he is currently 65 years of age or older. In accordance with 37 C.F.R. § 1.102(c), no fee should be required for entry and consideration of this Petition.

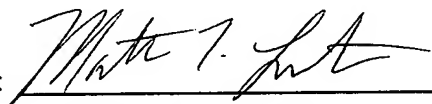
The currently pending claims, which have not been examined, are all directed to a single invention.

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A pre-examination search has been performed on the claims, as now pending. The results and search statements for the search are attached as part of an Appendix to this document, as are copies of the references deemed most relevant and a discussion of the references.

Respectfully submitted,
Cindy S. ORSER et al.

Date: 29 March 2006

By: 
Matthew T. Latimer
Reg. No. 44,204



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STATEMENT OF INVENTOR DAVIDSON REGARDING SPECIAL STATUS

Sir:

In connection with the Petition to Make Special filed herewith, I state that:

1. I am an inventor of the above-referenced patent application;
2. I am currently 75 years of age, having been born on May 27
1930
3. I believe that this Statement satisfies the requirements for a Statement of an inventor to support Special Status of this application, without the need for payment of a fee.

Respectfully submitted,
Eugene A. DAVIDSON

Date: 9/31/06

Eugene A. Davidson



APPENDIX - PETITION TO MAKE SPECIAL
10/728,246

- I. Fields of Search
- A. USPTO granted patents database:
1. - Keyword search (terms varied to create numerous statements, using ACLM and SPEC fields) for general concept:
prion\$, (detect\$ or identif\$ or determ\$), (assay or method)
 - Keyword search for particular concepts specifically recited in claims:
search strategies attached as "Keyword Search Terms"
 2. Classes/subclasses:
257/784; 257/741
422
424/520; 424/529; 424/530; 424/531
435/2; 435/4; 435/5; 435/7.1
436; 436/536; 436/543
455/561
514/2; 514/12; 514/13; 514/14; 514/15
530/300; 530/324; 530/326; 530/327; 530/350; 530/380
- B. USPTO patent application publications database
1. - Keyword search for general concept:
prion\$, (detect\$ or identif\$ or determ\$), (assay or method)
 - Keyword search for particular concepts specifically recited in claims:
search strategies attached as "Keyword Search Terms"
- C. EPO and WIPO Websites
1. - Keyword search for general concept:
prion\$, (detect\$ or identif\$ or determ\$), (assay or method)
 - Keyword search for general concepts specifically recited in title or abstract:
search strategies attached as "Keyword Search Terms"
- D. Medline and STN searches:
Keywords as detailed above, in numerous different permutations
- E. Dgene and nlm GenBank searches:
1. All SEQ ID Nos of application
 2. Re-search with VVAGAAAAGAMHKM

Keyword Search Terms

PGPs from USPTO website:

pyrene AND excimer AND (conformation OR conformational): 34 applications
ac1m/fluorescence AND ac1m/(protein OR prion) AND (conformation OR conformational) :
519 applications
ac1m/fluorescence AND ac1m/prion AND (conformation OR conformational): 11
applications
ac1m/fluorescence AND ac1m/(protein OR prion) AND ac1m/(detect OR detects OR
detection OR detecting OR detected): 1004 applications
ac1m/fluorescence AND ac1m/(prion) AND ac1m/(detect OR detects OR detection OR
detecting OR detected): 15 applications
ac1m/fluorescence AND (diagnostic OR diagnose OR diagnosis OR diagnosing) and prion
andnot ac1m/prion ANDNOT ac1m/(detect OR detects OR detection OR detecting OR
detected): 8 applications
ac1m/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect
OR detects OR detection OR detecting OR detected OR detector OR detective): 315
applications
ac1m/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect
OR detects OR detection OR detecting OR detected OR detector OR detective): 322
applications
ac1m/(conformation OR conformational) AND (prion) AND folding AND (detect OR detects
OR detection OR detecting OR detected OR detector OR detective): 38 applications
ac1m/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect
OR detects OR detection OR detecting OR detected OR detector OR detective): 322
applications

+++++

PATENTS from USPTO website:

pyrene AND excimer AND (conformation OR conformational): 44 patents
prion AND pyrene AND excimer AND (conformation OR conformational): 0 patents
ac1m/protein AND pyrene AND excimer AND (conformation OR conformational): 5 patents
protein ANDNOT ac1m/protein AND pyrene AND excimer AND (conformation OR
conformational): 23 patents
ac1m/fluorescence AND ac1m/(protein OR prion) AND (conformation OR conformational):
191 patents
ac1m/fluorescence AND ac1m/(protein OR prion) AND ac1m/(detect OR detects OR
detection OR detecting OR detected): 402 patents
ac1m/fluorescence AND ac1m/prion AND ac1m/(detect OR detects OR detection OR
detecting OR detected): 2 patents
ac1m/fluorescence AND prion ANDNOT ac1m/prion AND ac1m/(detect OR detects OR
detection OR detecting OR detected): 13 patents
ac1m/fluorescence AND (diagnostic OR diagnose OR diagnosis OR diagnosing) AND prion
ANDNOT ac1m/prion) ANDNOT ac1m/(detect OR detects OR detection OR detecting OR
detected): 3 patents
ac1m/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect
OR detects OR detection OR detecting OR detected OR detector OR detective): 141
patents

+++++

EPO website :

prion and (conformation or conformational) in the title or abstract : 29 hits
prion AND (detect or detection or detects or detected) in the title or abstract :
162 hits

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Keyword Search Terms

=> fil dgene

=> run getseq VVAGAAAAGAMHKM/sqsp : 3 hits

POTENTIALLY RELEVANT REFERENCES IDENTIFIED IN PRIOR ART SEARCH
10/728,246

Patent Publications:

USP 6,399,314 to Krishnamurthy

JP 2004-155688 to Numao

Non-Patent Publications:

Salmona, M., *et al.*, "Molecular determinants of the physicochemical properties of a critical prion protein region comprising residues 106-126", *Biochem. J.* **342**:207-214, 1999.

Summary of Currently Claimed Invention:

The present invention is directed to conformational probes that change conformation from alpha-helix and/or random coil to beta-sheet upon contact with a beta sheet structure in a target protein. In embodiments, the invention is a method for detecting β -sheet conformation of proteins or prions in a sample. The method comprises 1) reacting the sample with one or more α -helix or random coil conformational probes that interact with β -sheet conformation proteins or prions in the sample and thereby undergo a conformational conversion to a predominantly β -sheet conformation and form detectable aggregates with the β -sheet conformation proteins or prions in the sample, and 2) detecting levels of detectable aggregates. (See, for example, current claim 1.) In embodiments, the method is a method of diagnosing whether a subject suffers from or is predisposed to a disease associated with conformationally altered proteins or prions, in which the method comprises obtaining a sample from the subject and performing the method of the invention discussed above. (See, for example, current claim 60.) In other embodiments, the present invention is a kit for performing the method. (See, for example, present claim 33.) Further, in embodiments, the present invention is a conformational probe, such as one for practicing the methods of the invention. In embodiments, the probe comprises a first and third portion that are identical in sequence to a peptide sequence of a target protein that can assume a β -sheet conformation, and a second portion that is between the first and third portions, and which contains 1-10 residues, one of which is a proline. (See, for example, current claim 89.) In embodiments, the probe comprises residues of the Abeta protein of Alzheimer's Disease (see, for example, claim 104).

Summary of References Identified and Discussion of Patentability of Current Claims:

I. U.S. Patent No. 6,399,314 to Krishnamurthy discloses assays for detecting the amyloid protein of Alzheimer's Disease (AD), and in particular, the aggregation state (misfolded/aggregated vs. properly folded/soluble) of the protein. (See the '314 patent at col. 2, lines 27-48, for example.) The methods include binding an amyloid-specific spectroscopic probe (*i.e.*, Congo red) or a fluorophore-labeled amyloid peptide probe to an amyloid protein in a sample, and detecting the bound probe or peptide by its increased fluorescence when bound to the misfolded protein as compared to the properly folded protein. (See the '314 patent at col. 2, lines 39-67; col. 5, lines 20-63; col. 6, lines 33-60; and col. 7, lines 40-52, for example.) As the present claims relate to binding of peptide probes to conformationally altered or misfolded proteins, the disclosure of the '314 patent relating to binding of Congo red, which is not a peptide probe, is not relevant.

With regard to peptide probes, the '314 patent discloses that the method of the invention comprises adding a fluorophore-labeled amyloid peptide probe to an amyloid polypeptide mixture in a given aggregation state (already misfolded or already properly folded), and determining the relative spectroscopic properties of the probe. (See the '314 patent at col. 11, lines 30-35.) In certain embodiments, the probe may be added prior to the amyloid polypeptide achieving its final aggregation state. (See the '314 patent at col. 7, lines 31-39, for example.) Depending on the aggregation state and level of β -sheet structure of the amyloid polypeptide, the fluorophore-labeled amyloid peptide probe will show different properties (*e.g.*, higher UV emission when bound to misfolded/aggregated/ high β -sheet amyloid polypeptide, different far-UV CD spectra). (See the '314 patent at col. 6, lines 33-60, for example.)

In contrast to the presently claimed invention, which utilizes probes that undergo a conformational shift from alpha-helix and/or random coil to beta-sheet, at no point does the '314 patent disclose or suggest that the amyloid polypeptide changes the structure of the fluorophore-labeled amyloid peptide probe to convert it from a predominantly α -helix and/or random coil conformation to a predominantly β -sheet conformation. Thus, the '314 patent is not anticipatory art under 35 U.S.C. § 102 against the currently pending claims. Because the '314 patent does not suggest this concept or motivate one to achieve this concept, it likewise is not prior art against the currently pending claims under 35 U.S.C. § 103.

In view of the failure of the '314 patent to disclose or suggest a conformational probe according to the present claims, much less use of such a conformational probe to detect the presence of β -sheet conformation of proteins, the '314 patent fails to anticipate or render obvious the presently claimed invention.

II. Based on the English translation of Japanese patent publication JP 2004-155688 to Numao (attached), this patent publication discloses synthetic peptides having molecular chaperone activity (see '688 patent publication at first paragraph, "Problems to be Solved by the Invention"). The peptides comprise defined amino acid sequences and have peptide decarboxylation activity on oxalo-acetate (see '688 patent publication at second paragraph, "Means to Solve the Problem" and paragraph [0008]). The amino acid sequences are taken from, or derived from, sequences known in the art as being present in the prion protein at the region known to undergo conversion from alpha-helix or random coil in the normal cellular protein to

beta-sheet in the infectious protein. The peptides are said to have pharmaceutical properties (see, for example, paragraphs [0010] - [0021], for example).

While the '688 patent publication discusses the potential effects of peptides drawn from the same region of a prion protein as peptide probes of the current invention can be drawn, it fails to teach and/or suggest the presently claimed invention. More specifically, in contrast to the presently claimed invention, which uses probes to detect β -sheet conformation proteins or prions by detecting conversion of the conformation of the conformational probe from alpha-helix and/or random coil to beta-sheet, at no point does the '688 patent publication disclose or suggest this concept. Rather, at its most relevant, it discloses peptides taken from a region of a prion protein that is similar to the region serving as the basis for some peptide sequences for embodiments of the present invention, and suggests that those peptides can bind to proteins in a sample and protect them from decarboxylation. It does not, however, disclose or suggest that those peptides undergo any conformational change, or that such a change (if it were to occur) could be monitored to provide a method of detecting conformationally altered or misfolded proteins. Thus, the '688 patent publication is not anticipatory art under 35 U.S.C. § 102 against the currently pending claims. Furthermore, because the '688 patent publication does not suggest this concept or motivate one to achieve this concept, it is not prior art against the currently pending claims under 35 U.S.C. § 103.

III. Salmona *et al.* discloses the characterization of residues involved in conversion of the prion protein from a normal cellular protein to an infectious, misfolded protein (see Salmona abstract; page 208, first full paragraph). To do so, Salmona *et al.* made various substitutions in the prion protein sequence at residues 106-126, and tested the resulting peptides for alpha-helical characteristics (see Salmona, Experimental section, pages 208-209). In the Salmona *et al.* discloses that the peptides were capable of self-aggregation in various environments, regardless of their overall conformation (*i.e.*, alpha-helical or beta-sheet). However, self-aggregation was found to occur more readily for alpha-helical peptides (*e.g.*, peptides at pH 7.0). Salmona *et al.* propose that aggregates are formed in an anti-parallel fashion with a β -pleated sheet structure. (See Salmona *et al.* at page 213, first full paragraph and Figure 4).

With regard to patentability of the present invention over Salmona *et al.*, it is submitted that Salmona *et al.* does not disclose or suggest conformational probes, or use of such probes to detect the presence of β -sheet conformation of proteins or prions in a sample. Rather, Salmona *et al.* is completely focused on determination of residues that are involved in conversion of alpha-helical peptide structures to beta-sheet structures. Thus, Salmona *et al.* fails to teach and/or suggest the presently claimed invention. At its most relevant, it discloses that prion peptides comprising residues from the 106-126 region can self-aggregate, and that peptides that have an alpha-helical structure are more prone to do so. This is in contrast to the presently claimed invention, which utilizes probes that can aggregate upon conversion from α -helical or random coil structures to β -sheet structures.

Furthermore, Salmona *et al.* is completely silent with regard to conversion of an alpha-helical and/or random coil structure in a peptide to a predominantly beta-sheet structure upon contact of the peptide with a peptide or protein having a beta-sheet structure. Furthermore, it does not disclose that such a conversion should be monitored to provide a method of detecting conformationally altered or misfolded proteins.

Thus, Salmona *et al.* is not anticipatory art under 35 U.S.C. § 102 against the currently pending claims. Furthermore, because Salmona *et al.* does not suggest this concept or motivate one to achieve this concept, it is not prior art against the currently pending claims under 35 U.S.C. § 103.

(19) 日本国特許庁(JP)

(12) 公開特許公報(A)

(11) 特許出願公開番号

特開2004-155688

(P2004-155688A)

(43) 公開日 平成16年6月3日(2004.6.3)

(51) Int. Cl. ⁷	F I	テーマコード (参考)
C07K 7/08	C07K 7/08 ZNA	2G045
A61K 38/00	A61P 25/28	4C084
A61P 25/28	A61P 43/00 111	4H045
A61P 43/00	GO1N 33/15 Z	
GO1N 33/15	GO1N 33/50 Z	
審査請求 未請求 請求項の数 10 O L (全 22 頁) 最終頁に続く		
(21) 出願番号	特願2002-321436 (P2002-321436)	(71) 出願人 502154441
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(31) 優先権主張番号	特願2002-128976 (P2002-128976)	神奈川県相模原市西橋本5-4-21
(32) 優先日	平成14年4月30日 (2002.4.30)	(74) 代理人 100090273
(33) 優先権主張国	日本国 (JP)	弁理士 園分 孝悦
(31) 優先権主張番号	特願2002-200884 (P2002-200884)	(72) 発明者 沼尾 長徳
(32) 優先日	平成14年7月10日 (2002.7.10)	東京都町田市原町田5-2-17
(33) 優先権主張国	日本国 (JP)	Fターム (参考) 2G045 AA40 BB51 DA20 DA36 FB01
(31) 優先権主張番号	特願2002-268260 (P2002-268260)	CC10
(32) 優先日	平成14年9月13日 (2002.9.13)	4C084 AA02 AA06 BA01 BA08 BA18
(33) 優先権主張国	日本国 (JP)	BA23 CA59 DC50 NA14 ZA152
		ZA162 ZA182 ZC022
		4H045 AA10 AA30 BA16 EA20 EA50
		FA10

(54) 【発明の名称】 シャペロン活性を有する合成ペプチド、脱炭酸活性の測定方法、伝達性海綿状脳症用薬剤及びその探索方法

(57) 【要約】

【課題】 分子シャペロン活性を有する新規な合成ペプチドや伝達性海綿状脳症用薬剤及びその探索方法等を提供する。

【解決手段】 アミノ酸配列 Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X₁ (X₁ はASP又はGluである。) を少なくとも一部に有するペプチド、又は Ile-Ser-X₂-Gly-Ser-Gly-X₃-Thr-Trp-Ser-Asn-X₄-Thr (X₂、X₃ 及びX₄ はASP、Glu又はArgである。) を少なくとも一部に有するペプチドを、アリオン蛋白質のアミノ酸配列由来の Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly を少なくとも一部に有するペプチドに加えることによって、後者のペプチドの脱炭酸活性を観測することができる。

【選択図】 なし

【特許請求の範囲】

【請求項1】

下記のアミノ酸配列

Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X₁ (X₁はASP又はGluを表す。)を少なくとも一部に有することを特徴とするシャペロン活性を有する合成ペプチド。

【請求項2】

下記のアミノ酸配列

Ile-Ser-X₂-Gly-Ser-Gly-X₃-Thr-Trp-Ser-Asn-X₄-Tyr又は

Tyr-X₄-Asn-Ser-Trp-Thr-X₃-Gly-Ser-Gly-X₂-Ser-Ile

(X₂、X₃及びX₄はASP、Glu又はArgである。)を少なくとも一部に有することを特徴とするシャペロン活性を有する合成ペプチド。

【請求項3】

前記アミノ酸配列のN末端がNH₂又はNHCOCH₃であり、C末端がCOOH又はCONH₂であることを特徴とする請求項1又は2に記載のシャペロン活性を有する合成ペプチド。

【請求項4】

下記のアミノ酸配列

Ile-Ser-X₂-Gly-Ser-Gly-X₃-Thr-Trp-Ser-Asn-X₄-Tyr又は

Tyr-X₄-Asn-Ser-Trp-Thr-X₃-Gly-Ser-Gly-X₂-Ser-Ile

(X₂、X₃及びX₄はASP、Glu又はArgである。)を少なくとも一部に有するペプチドを、トリフロロエタノールとオキサロアセテートを含む緩衝液に加える工程を有することを特徴とする脱炭酸活性の測定方法。

【請求項5】

フリオン蛋白質のアミノ酸配列由来の下記のアミノ酸配列

Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly又は

Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys

を少なくとも一部に有するペプチドを、請求項1乃至3のいずれか1項に記載の合成ペプチドとオキサロアセテートを含む緩衝液に加える工程を有することを特徴とする脱炭酸活性の測定方法。

【請求項6】

前記アミノ酸配列のN末端をNH₂又はNHCOCH₃とし、C末端をCOOH又はCONH₂とすることを特徴とする請求項4又は5に記載の脱炭酸活性の測定方法。

【請求項7】

請求項1乃至3のいずれか1項に記載の合成ペプチドを用いたことを特徴とする伝達性海綿状脳症用薬剤。

【請求項8】

フリオン蛋白質のアミノ酸配列由来の下記のアミノ酸配列

Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly又は

Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-

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Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys

を少なくとも一部に有するペプチドを用いることを特徴とする伝達性海綿状脳症用薬剤の探索方法。

【請求項 9】

前記アミノ酸配列のN末端をNH₂又はNHCOCH₃とし、C末端をCOOH又はCONH₂とすることを特徴とする請求項 8 に記載の伝達性海綿状脳症用薬剤の探索方法。

【請求項 10】

塩基性アミノ酸リジン残基を少なくとも1個含む両親媒性アミノ酸配列を用いることを特徴とする伝達性海綿状脳症用薬剤の探索方法。

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【発明の詳細な説明】

【0001】

【発明の属する技術分野】

本発明は、プリオン蛋白質の部分アミノ酸配列（セグメント）に対してシャペロン活性を有する新規な合成ペプチド、脱炭酸活性の測定方法、伝達性海綿状脳症用薬剤及びその探索方法に関する。

【0002】

【従来の技術】

伝達性海綿状脳症（クロイツフェルト・ヤコブ病、スクレイビー、牛海綿状脳症など）の治療薬の開発には、社会からの強力な要請がある。これまでに幾つかの低分子化合物が開発されてきているが、効力性と副作用の点から、より優れた活性をもつ化合物の開発が望まれている（K. T. Adjou et al., CNS Drugs 10, 83-89 (1998)）。

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【0003】

伝達性海綿状脳症と高分子シャペロン（Protein X）との関連性は既に指摘されているが、高分子シャペロンの単離同定には到っていない（F. E. Cohen & S. B. Prusiner, Annu. Rev. Biochem., 67, 793-819 (1998)）。但し、13個のアミノ酸残基からなる合成ペプチド（iPrP13）によるヒト（又はマウス）感染性プリオン蛋白質（PrP^{Sc}）の正常プリオン蛋白質（PrP^C）への変換が既に報告されている（C. Soto et al., Lancet 355, 192-197 (2000), C. Soto et al., Biochem. Biophys. Chem. Commun., 226, 672-680 (1996)）。

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【0004】

所望の分子シャペロン活性を有する短鎖ペプチド又は低分子有機化合物を開発するためには、生物活性を有する既存のアミノ酸配列中のアミノ酸残基を他のアミノ酸残基に置換するという方法や自然界からのランダムスクリーニング方法が採用される。例えば、前者の方法では、その配列中のアミノ酸残基の親媒性に着目して、似たような性質をもつほかのアミノ酸残基と入れ替える。また、N末端のアセチル化、C末端のアミド化、あるいはペプチド結合（-CONH-）を-CH₂-NH-、-CH=CH-、-NHCO-のように変換したり、適当な位置に2つのシステイン残基を導入して直線型から環状型にしたり、あるいはその逆方向（環状型から直線型）へ変換したり、またL-アミノ酸残基をD-アミノ酸残基と交換したりする改変方法もある。更に、新しい1つの合成方法として、天然型アミノ酸配列の逆配列合成法もある（B-L. Lie et al., Biol. Pharm. Bull., 19, 1602-1606 (1996)）。

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【0005】

そのような既知手段の中で、Soto らはiPrP13の逆配列ペプチドに関する分子シャペロン活性を全く言及していない（C. Soto et al., Lancet 355, 192-197 (2000), C. Soto et al., Biochem. Biophys. Chem. Commun., 226, 672-

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680 (1996))。

【0006】

感染性プリオン蛋白質 (PrP^{Sc}) と伝達性海綿状脳症との関連性は多くの研究者によって議論されているが、正常なプリオン蛋白質 (PrP^C) については、銅結合蛋白質やストレス応答蛋白質以外の機能は不明である。但し、プリオン蛋白質の部分ペプチドがトリフクロエタノール存在下、オキサロアセテートに対して脱炭酸活性を発現するかもしれないことが推測されているが実証されていない (特開2002-22736号公報)。

【0007】

【発明が解決しようとする課題】

本発明の目的は、分子シャペロン活性を有する新規なシャペロン活性を有する合成ペプチド、脱炭酸活性の測定方法、伝達性海綿状脳症用薬剤及びその探索方法を提供することである。

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【0008】

【課題を解決するための手段】

本発明者は鋭意検討した結果、プリオン蛋白質のアミノ酸配列由来のアミノ酸配列 R^4 NH-LYS-THR-ASN-MET-LYS-HIS-MET-ALA-GLY-ALA-ALA-ALA-GLY-ALA-VAL-VAL-GLY-GLY-LEU-GLY-COR⁵ (配列表中の配列番号13) 又は R^4 NH-GLY-LEU-GLY-GLY-VAL-VAL-ALA-GLY-ALA-ALA-ALA-ALA-GLY-ALA-MET-HIS-LYS-MET-ASN-THR-LYS-COR⁵ (配列表中の配列番号14) (R^4 は水素原子又はアセチル基であり、 R^5 はOH又はNH₂である。) で表されるアミノ酸配列の何れか1つが、トリフクロエタノール (TFE) 存在下で、オキサロアセテートの脱炭酸活性を促進することを見出し、更にそのアミノ酸配列はアミノ酸配列 R^1 NH-VAL-PRO-VAL-ALA-PRO-GLY-ALA-PRO-ALA-ALA-PRO-ALA-X₁-COR² (配列表中の配列番号10) (X_1 はASP又はGLUであり、 R^1 は水素原子又はアセチル基であり、 R^2 はOH又はNH₂である。) で表されるペプチドが、又は R^3 NH-ILE-SER-X₂-GLY-SER-GLY-X₃-THR-TRP-SER-ASN-X₄-TYR-COR⁴ (配列表中の配列番号11) 若しくは R^3 NH-TYR-X₄-ASN-SER-TRP-THR-X₃-GLY-SER-GLY-X₂-SER-ILE-COR⁴ (配列表中の配列番号12) (X_2 、 X_3 及び X_4 はASP、GLU又はALAであり、 R^1 は水素原子又はアセチル基であり、 R^2 はOH又はNH₂である。) で表されるペプチドのうち何れか1つのペプチド又はクロルフロマジンを加えると、TFE非存在下でも、オキサロアセテートの脱炭酸活性を促進することを見出し、本発明を完成させた。

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【0009】

更に好ましい態様としては、 X_1 はASP、 X_2 、 X_3 及び X_4 はASP又はALAである。

【0010】

また、本願発明に係る伝達性海綿状脳症用薬剤の探索方法は、上記のいずれかの合成ペプチドを用いるか、又はカルシトニン等の塩基性アミノ酸リジン残基を少なくとも1個含む両親媒性アミノ酸配列を用いることを特徴とする。

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【0011】

【発明の実施の形態】

本発明のペプチドは活性化エステル法、混合酸無水物法、アジド法などのC端活性化法、カルボジミドなどのカップリング法、N-カルボキシ無水物 (NCA) 法、酸化還元法あるいは固相合成法等の方法により合成することができる。

【0012】

本発明のペプチドの有効成分として含む分子シャペロン剤においては該ペプチドに代えてあるいは該ペプチドと共に、上記のペプチドの生理学的に許容される塩を有効成分として含んでいてもよく、生理学的に許容される塩としてはアルカリ、無機酸または有機酸、例

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えは水酸化ナトリウム、水酸化カルシウム、水酸化マグネシウム、水酸化カリウム、塩酸、硫酸、磷酸、酢酸、クエン酸、酒石酸、乳酸、オレイン酸、フマル酸等との塩を挙げることができる。

【0013】

本発明におけるペプチドまたはその塩は治療または予防のための経口的あるいは非経口的に投与することができる。

【0014】

経口投与剤としては散剤、粒剤、カプセル剤、錠剤などの固形製剤あるいはシロップ剤、エリキシル剤などの液状製剤とすることができる。また、非経口投与剤としては注射剤、直腸投与剤、皮膚外用剤、吸入剤とすることができる。これらの製剤は活性成分に薬学的に認容できる製造助剤を加えることにより常法に従って製造される。更に、公知の技術により持続性製剤とすることも可能である。

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【0015】

経口投与用の固形製剤を製造するには活性成分と賦形剤、例えば乳糖、澱粉、結晶セルロース、乳酸カルシウム、メタケイ酸アルミン酸マグネシウム、無水ケイ酸などと混合して散剤とするか、更に必要に応じて白糖、ヒドロキシプロピルセルロース、ポリビニルピロリドンなどの結合剤、カルボキシメチルセルロース、カルボキシメチルセルロースカルシウムなどの崩壊剤などを加えて湿式または乾式造粒して粒剤とする。錠剤を製造するにはこれらの散剤及び粒剤をそのままあるいはステアリン酸マグネシウム、タルクなどの滑沢剤を加えて打錠すればよい。これらの粒または錠剤はヒドロキシメチルセルロースフタレート、メタアクリル酸、メタアクリル酸メチルコポリマーなど腸溶性基剤で被覆して腸溶性製剤、あるいはエチルセルロース、カルナウバロウ、硬化油などで被覆して持続性製剤とすることもできる。また、カプセル剤を製造するには散剤又は粒剤などの硬カプセルに充填するか、活性成分をグリセリン、ポリエチレングリコール、ゴマ油、オリーブ油などに溶解したのちゼラチン膜で被覆し軟カプセルとすることができる。

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【0016】

経口投与用の液状製剤を製造するには活性成分と白糖、ソルビトール、グリセリンなどの甘味剤とを水に溶かして透明なシロップ剤、更に精油、エタノールなどを加えてエリキシル剤とするか、アラビアゴム、トラガント、ポリソルベート80、カルボキシメチルセルロースナトリウムなどを加えて乳剤または懸濁剤としてもよい。これらの液状製剤には所望により矯味剤、着色剤、保存剤などを加えてもよい。

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【0017】

注射剤を製造するには活性成分を必要に応じて塩酸、水酸化ナトリウム、乳酸、乳酸ナトリウム、リン酸一水素ナトリウム、リン酸二水素ナトリウムなどのPH調整剤、塩化ナトリウム、ブドウ糖などの等張化剤とともに注射用蒸留水に溶解し、無菌ろ過してアンフルに充填するか、更にマンニトール、デキストリン、シクロデキストリン、ゼラチンなどを加えて真空下凍結乾燥し、用事溶解型の注射剤としてもよいし、活性成分にレシチン、ポリソルベート80、ポリオキシエチレン硬化麻子油などを加えて水中で乳化せしめ注射用乳剤とすることもできる。

【0018】

直腸投与剤を製造するには活性成分及びカカオ脂、脂肪酸のモノ、ジ及びトリグリセリド、ポリエチレングリコールなどの坐剤用基剤とを加湿して溶融し、型に流し込んで冷却するか、活性成分をポリエチレングリコール、大豆油などに溶解したのちゼラチン膜で被覆すればよい。

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【0019】

皮膚外用剤を製造するには活性成分を白色ワセリン、ミツロウ、流動パラフィン、ポリエチレングリコールなどに加えて必要ならば加湿して練合し軟膏剤とするか、ロジン、アクリル酸アルキルエステル重合体などの粘着剤と練合したのちポリエチレンなどの不織布に展延してテープ剤としてもよい。

【0020】

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吸入剤を製造するには活性成分をフロンガスなどの噴射剤に溶解または分散して耐圧容器に充填しエアゾール剤としてもよい。

【0021】

本発明のペプチドの投与量は伝達性海綿状脳症の患者（牛、羊などの動物も含め）の年齢、体重及び病態に依ってこととなるが、通常一日当たり約1～500mgであり、1乃至数回に分けて投与することが望ましい。

【0022】

以下に、本発明について、実施例に基づいて具体的に説明するが、本発明はこれらに限定されるものではない。また、本活性測定方法を全種アリオン蛋白質の全長アミノ酸配列に応用したり、アミノ酸親媒性などの分類法に関する既知知見を用いて蛋白質データベース等から本発明のシャペロン活性を有する合成ペプチドのアミノ酸配列と相同性の高い（40%以上）配列を持つ蛋白質を探索し、その相同性の高いアミノ酸配列を含む該蛋白質のアミノ酸配列全体又は部分を同一目的（例えば、伝達性海綿状脳症の治療、予防又は検査薬の開発）に利用することも容易に類推でき、本発明範囲に含まれる。更に、本活性測定法は、伝達性海綿状脳症の治療、予防又は検査用の薬剤の探索方法にも利用できることは明白であり、その用途法が本実施例に限定されるものではない。

【0023】

【実施例】

先ず、種々の合成ペプチドを準備した。本実施例に用いた合成ペプチドは下記表1に示すとおりである。

【0024】

【表1】

配列番号	アミノ酸配列
1	GIGKFLKKAKKFAKAFVKILKK-CONH ₂
2	LAKLLKALAKLLKK-CONH ₂
3	KTNMKHMAGAAAAGAVVGGLG-COOH
4	GLGGVVAGAAAAGAMHKMNTK-COOH
5	DAPAAPAGPAVPV-COOH
6	VPVAPGAPAAPAD-COOH
7	ISRGSGRTWSNRY-COOH
8	ISDGSGDTWSNDY-COOH
9	CH ₃ CONH-KTNMKHMAGAAAAGAVVGGLG-COOH

【0025】

配列番号1、2、3及び5に記載のアミノ酸配列は公知である（A. Iwakori et al., Biol. Pharm. Bull. 20, 267-270 (1997); K. Johansson et al., Nature 365, 530-532 (1993); G. Forloni et al., Nature 362, 543-546 (1993); C. Soto et al., Lancet 355, 192-197 (2000)）。一方、配列番号4、6、7、8及び9に記載のアミノ酸配列は本発明範囲に含まれるものである。また、配列番号1、2、3、4及び9に記載のアミノ酸配列は、塩基性アミノ酸リジン残基を少なくとも1個含む両親媒性アミノ酸配列であり、このような両親媒性アミノ酸配列としては、他にカルシトニンが挙げられる。

【0026】

次に、脱炭酸酵素活性測定を行った。この測定方法は以下のとおりである。2.98 mM オキサロアセテート (1.7 ml: 50 mM MOPS, 0.15 M NaCl, PH 7.5) とトリフロロエタノール (TFE) (0.2 ml) を分光器セルに加え、室温で、5 分間 した。後、その溶液に 2.0 mM 濃度の測定用サンプルを 0.1 ml 加えて反応溶液全量を 2 ml とし、室温で、1 分間 (Iuchi, HS-3B, 回転スピード 4) した。その後、 を停止し、分光器 (UltraoSpec 3100 Pro, Amersham Biosciences corp.) で 285 nm の吸光度を測定した。対照サンプルとしては 2.0 mM 配列番号 2 (0.2 mM) を用いた。

【0027】

反応開始後、2500 秒後の配列番号 2 に記載のアミノ酸配列存在下での吸光度減少量 a を求めた。同様に、配列番号 2 に記載のアミノ酸配列非存在下でのオキサロアセテートの吸光度減少量 b を求めた。試験ペプチド (配列番号 1, 3, 4, 5, 6, 7 及び 8) についても同様に吸光度減少量 c を求め、下記の数式 1 により試験ペプチドの配列番号 2 に記載のアミノ酸配列に対する相対比活性を求めた。

【0028】

【数 1】

$$\text{相対比活性} = (c - b) / (a - b)$$

【0029】

この測定結果を表 2 に示す。

【0030】

【表 2】

配列番号	相対比活性
1	0.32
2	1.00
3	0.12
4	0.12
5	0.02
6	0.04
7	NT
8	NT
9	NT

【0031】

この結果より配列番号 3 及び 4 に記載のアミノ酸配列は TFE 存在下脱炭酸活性を有していることが明らかとなった。

【0032】

次に、合成ペプチドのシャペロン活性測定を行った。この測定方法は以下のとおりである。2.98 mM オキサロアセテート (1.7 ml: 50 mM MOPS, 0.15 M NaCl, PH 7.5) と TFE (0.2 ml) を分光器セルに加え、室温で、5 分間 した。後、その溶液に 2.0 mM 濃度の測定用サンプルを 0.1 ml 加えて反応溶液全量を 2 ml とし、室温で、1 分間 (Iuchi, HS-3B, 回転スピ

ード4)した。その後、を停止し、分光器(Ultraviolet Spec 3100 Pro, Amersham Biosciences corp.)で285nmの吸光度を測定した。対照サンプルとしては2.0mM配列番号3(又は4)に記載のアミノ酸配列(0.2mM)を用いた。反応開始後、2500秒後の配列番号3に記載のアミノ酸配列存在下での吸光度減少量dを求めた。同様にして、配列番号3に記載のアミノ酸配列非存在下でのオキサロアセテートの吸光度減少量eを求めた。

【0033】

次に、上記操作のTFEの代わりに試験ペプチド(配列番号5、6、7又は8)を0.2ml加え、室温で5分間した。後、その溶液に2.0mM濃度の測定用サンプル(配列番号3)を0.1ml加えて反応溶液全量を2mlとし、室温で、1分間(Iucci, HS-3B, 回転スピード4)した。を停止後、分光器で285nmの吸光度減少量fを測定した。下記の数式2により試験ペプチドの配列番号3(又は4)に記載のアミノ酸配列に対する相対比活性を求めた。

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【0034】

【数2】

$$\text{相対比活性} = (f - e) / (d - e)$$

【0035】

この測定結果を表3に示す。

【0036】

【表3】

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試料	相対比活性
配列番号 3 (200 μ M) + TFE	1.00
配列番号 4 (200 μ M) + TFE	1.00
配列番号 3 (200 μ M) + 配列番号 5 (100 μ M)	0.12
配列番号 3 (200 μ M) + 配列番号 5 (200 μ M)	0.38
配列番号 3 (200 μ M) + 配列番号 6 (100 μ M)	0.42
配列番号 3 (200 μ M) + 配列番号 6 (200 μ M)	0.44
配列番号 3 (200 μ M) + 配列番号 7 (100 μ M)	NT
配列番号 3 (200 μ M) + 配列番号 7 (200 μ M)	NT
配列番号 3 (200 μ M) + 配列番号 8 (100 μ M)	NT
配列番号 3 (200 μ M) + 配列番号 8 (200 μ M)	NT

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【0037】

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この結果より、本発明のペプチド（配列番号 6）はシャペロン活性を有していることが明らかとなった。

【0038】

更に、他の方法により、合成ペプチドのシャペロン活性測定を行った。この測定方法は以下のとおりである。分光器セル中、2 mM 配列番号 3（又は 4）（0.2 ml）、緩衝液（50 mM MOPS, 0.15 M NaCl, PH7.0）（1.4 ml）の混合溶液に、2 mM 測定用サンプル（配列番号 5, 6, 7, 8）（0.2 ml）を加え、室温で 48 時間（Iuchi, HS-3B, 回転スピード 4）した。後、2.98 mM オキサロアセテート（0.2 ml）を加えて全量を 2.0 ml とし、更に 1 分間した。を停止し、分光器（UltraSpec 3100 Pro, Amersham Biosciences corp.）で 285 nm の吸光度減少量を反応開始後 5000 秒間測定した。対照サンプルとしては、測定用サンプル非存在下、トリフロロエタノール（TFE）存在下の配列番号 3 で測定した吸光度減少量を 1.00 とした。

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【0039】

この測定結果を表 4 に示す。

【0040】

【表 4】

試料	相対比活性
配列番号 3 (200 μ M) + TFE (+)	1.00
配列番号 3 (200 μ M) + TFE (-)	0.59
配列番号 4 (200 μ M) + TFE (+)	0.70
配列番号 4 (200 μ M) + TFE (-)	0.54
配列番号 3 (200 μ M) + 配列番号 5 (200 μ M)	0.62
配列番号 3 (200 μ M) + 配列番号 6 (200 μ M)	0.60
配列番号 3 (200 μ M) + 配列番号 7 (200 μ M)	0.65
配列番号 3 (200 μ M) + 配列番号 8 (200 μ M)	0.57

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【0041】

この結果より、本発明のペプチド（配列番号 6 及び 7）はシャペロン活性を有していることが明らかとなった。

【0042】

次に、上記の方法とは異なる方法により、脱炭酸活性測定を行った。この測定方法は以下のとおりである。分光器セル中、2 mM 配列番号 9 (0.2 ml)、緩衝液 (50 mM MOPS, 0.15 M NaCl, PH 7.0) (1.4 ml) の混合溶液に、TFE (0.2 ml) を加え、室温で 48 時間 (Luci, HS-3B, 回転スピード 4) した。後、2.98 mM オキサロアセテート (0.2 ml) を加えて全量を 2.0 ml とし、更に 1 分間 した。を停止し、分光器 (UltraSpec 3100 Pro, Amersham Biosciences corp.) で 285 nm の吸光度減少量を反応開始後 5000 秒間測定した。対照サンプルとしては、測定用サンプル非存在下、トリフクロエタノール (TFE) 存在下の配列番号 2 で測定した吸光度減少量を 1.00 とした。

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【0043】

この測定結果を表 5 に示す。

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【0044】

【表 5】

試料	活性	相対比活性
配列番号 2 (200 μ M) + TFE (+)	0.195	1.00
配列番号 2 (200 μ M) + TFE (-)	0.159	
配列番号 9 (200 μ M) + TFE (+)	0.169	0.61
配列番号 9 (200 μ M) + TFE (-)	0.147	

【0045】

この結果より、配列番号 9 に記載のアミノ酸配列は TFE 存在下で脱炭酸活性を有していることが明らかとなった。

【0046】

次に、抗精神病薬等として使用されるクロルプロマジンのシャペロン活性測定を行った。この測定方法は以下のとおりである。分光器セル中、2 mM 配列番号 9 (0.2 ml)、緩衝液 (50 mM MOPS, 0.15 M NaCl, pH 6.0) (1.4 ml) の混合溶液に、2 mM 測定用サンプル (例えば、クロルプロマジン) を加え、室温で 48 時間 (Uichi, HS-3B, 回転スピード 4) した。後、2.98 mM オキサロアセテート (0.2 ml) を加えて全量を 2.0 ml とし、更に 1 分間 した。

を停止し、分光器 (UltraSpec 3100 Pro, Amersham Biosciences corp.) で 285 nm の吸光度減少量を反応開始後 5000 秒間測定した。対照サンプルとしては、配列番号 9 非存在下、探索用サンプル (クロルプロマジン) 存在下で測定した吸光度減少量を 1.00 とした。

【0047】

この測定結果を表 6 に示す。

【0048】

【表 6】

試料	相対比活性
クロルプロマジン (200 μ M) (pH 6.0)	1.00
緩衝溶液中でのオキサロアセテートの自己分解 (pH 6.0)	1.00
配列番号 9 (200 μ M) + クロルプロマジン (200 μ M) (pH 6.0)	1.31

【0049】

この結果より、本発明のシャペロン活性測定法は伝達性海綿状脳症用治療薬としての低分子有機化合物を探索できることが明らかとなった。

【0050】

【発明の効果】

上記ペプチドは分子シャペロン活性を有し、伝達性海綿状脳症の治療、予防又は検直薬としての用途を有する。

【0051】

【配列表】

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<110> BioFrontier Institute INC.

<120> Synthetic Peptide with Chaperone Activity

<130> XY04651

<140>

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<141>

<150> JP 2002-128976

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(220)

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(222) (2)

(223) Xaa represents Asp, Glu or Arg

10

(220)

(221) PEPTIDE

(222) (7)

(223) Xaa represents Asp, Glu or Arg

(220)

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(221) PEPTIDE

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(223) Xaa represents Asp, Glu or Arg

(400) 12

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[Problems to be Solved by the Invention]

To provide a new synthetic peptide having a molecular chaperone activity, to provide a pharmaceutical for transmissible spongiform encephalopathy, to provide a method for researching the same, and the like.

[Means to Solve the Problems]

Amino acid sequence Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X(1) [where X(1) is Asp or Glu] peptide, or Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr [where X(2), X(3) and X(4) are Asp, Glu or Arg]. Peptide at least in part adds to peptide which comprises Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly of the amino acid sequence derived from prion protein, such that decarboxylation activity of peptide of the latter can be observed.

[Selected Drawing] none

[Claims]

[Claim 1]

A synthetic peptide comprising the sequence: Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X(1) where X(1) is Asp or Glu, where the synthetic peptide possesses chaperone activity.

[Claim 2]

A synthetic peptide comprising the sequence: Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr or Tyr-X(4)-Asn-Ser-Trp-Thr-X(3)-Gly-Ser-Gly-X(2)-Ser-Ile, where X(2), X(3) and X(4) are Asp, Glu or Arg, where the synthetic peptide possesses chaperone activity.

[Claim 3]

The N-terminal portion of aforementioned amino acid sequence beginning with NH₂ or NHCOCH₃, in Claim 1 or 2, with C-terminal being COOH or CONH₂, and where the stated synthetic peptide possesses chaperone activity.

[Claim 4]

An amino acid sequence comprising: Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr or Tyr-X(4)-Asn-Ser-Trp-Thr-X(3)-Gly-Ser-Gly-X(2)-Ser-Ile where X(2), X(3) and X(4) are Asp, Glu or Arg, which may be used for purposes of a measurement method, to measure decarboxylation activity, when added to a buffer which includes trifluoro-ethanol and oxalo-acetate.

[Claim 5]

A peptide having an amino acid sequence derived from a prion protein, comprising: Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly or Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys, which may be used for purposes of a measurement method, to measure decarboxylation activity when added to a buffer which includes synthetic peptide and oxalo acetate which stated peptide comprises at least in part, the peptides stated in the any one claim of Claims 1 through 3.

[Claim 6]

A measurement method for measuring decarboxylation activity, as stated in Claim 4 or 5, using peptide with a designated N-terminal of aforementioned amino acid sequence as being NH₂ or NHCOCH₃, and with designated C-terminal as being COOH or CONH₂.

[Claim 7]

A pharmaceutical drug for transmissible spongiform encephalopathy, designated as the synthetic peptide stated in any one claim of Claims 1 through 3.

[Claim 8]

An amino acid sequence derived from the amino acid sequence prion protein, comprising the sequence Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly or Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys, used for purposes of a research method for screening pharmaceutical drugs for transmissible spongiform encephalopathy, designating any peptide which possesses such sequence at least in part.

[Claim 9]

A research method for screening pharmaceutical drugs for transmissible spongiform encephalopathy, as stated in Claim 8, which peptide is designated to have an N-terminal of aforementioned amino acid sequence as being NH₂ or NHCOCH₃, and a C-terminal as being COOH or CONH₂.

[Claim 10]

A research method for screening pharmaceutical drugs for transmissible spongiform encephalopathy, designating an amphipathic amino acid sequence which includes a basic amino acid such as a lysine residue as at least one of the amino acid residues.

[Description of the Invention]

[0001]

[Technological Field of Invention]

This invention relates to a pharmaceutical drug for transmissible spongiform encephalopathy and a research method for measuring decarboxylation activity of novel synthetic peptide which possesses chaperone activity, comprising a partial amino acid sequence of prion protein.

[0002] [Prior Art]

There is strong request from society for development of a treatment drug for transmissible spongiform encephalopathy such as Creutzfeldt-Jakob illness [sukureipii], or bovine spongiform encephalopathy. (K.T. Adjou et al., CNS Drugs 10:83-89 [1998]). Several low-molecular weight compounds have been developed so far, but it is desired that compounds with such activity have better effectiveness and fewer side effects.

[0003]

As described by F.E.Cohen and S.B.Prusiner [Annu. Rev. Biochem. 67:793-819 (1998)], transmissible spongiform encephalopathy may involve a polypeptide chaperone (protein X), or polymeric form of the chaperone [C. Soto et al., Lancet 355(9199):192-7 (2000); C. Soto et al., Biochem Biophys Res Commun. 226(3):672-80 (1996)]. Furthermore, conversion from normal form of human (or mouse) prion protein (PrP_c) to the contagious form (PrP_{Sc}) is reported using a synthetic peptide (iPrP-13) consisting of 13 amino acid residues.

[0004]

In order to develop a short chain peptide of low molecular weight as an organic compound which possesses desired molecular chaperone activity, a random screening method was adopted, with substituting an amino acid residue within an existing amino acid sequence which possesses biological activity. With using methods from earlier studies, paying attention to characteristics of amino acid residues, such methods replaced an amino acid residue with another which has similar properties. In addition, there are also alternative methods using -CH(2)-NH-, -CH=CH-, -NHCO- to produce conversion by amidation reaction, to produce acetylation of the peptide bond (-CONH-), or acetylation of the C-terminal or the N-terminal, or by introducing cysteine residues into suitable location, changing the linear structure to a cyclic structure, or from cyclic to linear type, in addition by exchanging L- amino acid residues with D- amino acid residues, or by constructing inverse sequence peptides. [B.L Lie et al., Biol Pharm Bull. 19(12):1602-6 (1996)].

[0005]

For example, as reported by C. Soto et al (op. cit.) such methods can be used to produce peptides that block conversion to the beta-sheet conformation.

[0006]

The relevance of contagious prion protein (PrP_{Sc}) to transmissible spongiform encephalopathy is argued by many researchers, but a precise connection with normal prion protein (PrP_c) function other than via the stress response proteins remains unclear. However, from studies of synthetic peptides made from the prion protein using trifluoro-ethanol, it is presumed (although not proven) that such connection might reveal decarboxylation activity vis-a-vis the oxalo-acetate (Japan Unexamined Patent Publication 2002-22736 disclosure).

[0007]

[Problems to be Solved by the Invention]

An objective of this invention is to provide a compound for a method of measuring [or detecting] transmissible spongiform encephalopathy using a synthetic peptide, by measuring decarboxylation activity concomitant with molecular chaperone activity, and to provide a research method for same.

[0008]

[Means to Solve the Problems]

A peptide discovered by this inventor, as the result of diligent investigation, comprises the amino acid sequence R4NH-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly-COR5 as derived from the amino acid sequence of prion protein (Sequence Number 13 in sequence table) or R4NH-Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys-COR5 (Sequence Number 14 in sequence table) (where R4 denotes hydrogen atom or acetyl group, and R5 denotes OH or NH₂) where either of these amino acid sequences is shown by using measures of trifluoro-ethanol (TFE), to determine whether decarboxylation activity of oxalo-acetate is promoted. Furthermore, as for amino acid sequence amino acid sequence R1NH-Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X(1)-COR2 (Sequence Number 10 in sequence table) (where X(1) denotes Asp or Glu, and where R1 denotes hydrogen atom or acetyl group, and where R2 denotes OH or NH₂), or as for R3NH-Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr-COR4 (Sequence Number 11 in sequence table) or R3NH-Tyr-X(4)-Asn-Ser-Trp-Thr-X(3)-Gly-Ser-Gly-X(2)-Ser-Ile-COR4 (Sequence Number 12 in sequence table) (where X(2), X(3) and X(4) denotes Asp, Glu or Arg, and where R1 denotes hydrogen atom or acetyl group, and where R2 denotes OH or NH₂). For any of the foregoing peptides, as compared with added chlopromazine, it can be determined whether decarboxylation activity of oxalo-acetate is promoted, which can be discovered even with the absence of TFE, by which discovery this invention was completed.

[0009]

Furthermore, X(1) may denote Asp, and X(2), X(3) and X(4) may denote Asp or Arg, as additional desirable embodiments.

[0010]

In addition, a research method for drugs for transmissible spongiform encephalopathy relates to the invention of this application, using any of the above-mentioned synthetic peptides, or designating that an amphipathic amino acid sequence as for example which occurs in calcitonin, with at least one or more basic amino acid lysine residues.

[0011]

[Embodiment of the Invention]

The synthesis of peptides according to this invention may be accomplished using an activated ester method, mixed acid anhydride method, azide method or other C-terminus activation method, carbodiimide or other coupling method, or an N-carboxy anhydride (NCA) method, with an oxidation and reduction method or solid phase synthesis method or other method.

[0012]

Replacing the said peptide as active ingredient with the peptide of this invention concerns a molecular chaperone agent which it includes, or which combines with said peptide, by means of which it is possible to include a physiologically acceptable salt of the above-mentioned peptide, as the active ingredient as physiologically acceptable salt, or alkali inorganic acid or organic acid, for example sodium hydroxide, calcium hydroxide, magnesium hydroxide, potassium hydroxide, hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, citric acid, tartaric acid, lactic acid, oleic acid, fumaric acid, etc.

[0013]

The prescribed peptide or its salt may, according to this invention, be made for oral or parenteral delivery, and for treatment or prevention.

[0014]

The prescribed peptide can be made into a powder, granule, capsule, tablet or other solid preparation or syrup, elixir or other liquid state for formulation as an oral dosage agent. In addition, it can be made injectable, for rectal administration, for an external skin preparation, for an inhalant, or for parenteral administration. These formulations following conventional methods, by adding manufacturing processes by which the active ingredient in pharmacological may be produced. Furthermore, it is also possible to make an increased retention formulation, with known technology.

[0015]

A solid preparation for oral dosage is produced, using active ingredient and a vehicle, for example lactose, starch, crystalline cellulose, calcium lactate, sodium metasilicate aluminate magnesium, anhydrous silicic acid, etc; and by mixing or making a powder, or with wet type or dry type granulation using sucrose [hidorokishi puropiru seruroosu], poly vinyl pyrrolidone chain or other binder [karuboki shimechiru seruroosu], including [karubokishi mechiruseruro osukarushiumu] or other disintegrating granulating agent to make tablets. These powder and granule pill-making processes must be done using magnesium stearate ** [or other substances], including talc or other lubricant. The granule or tablet sheath may be made with an enteric base, such as hydroxymethyl cellulose phthalate, methacrylic acid, methyl methacrylate copolymer, or made with other enteric sheath formulation, such as ethyl cellulose, carnauba wax, or hydrogenated oil to make an increased retention formulation. In addition, capsules produced may be filled with powder, or with granules or other hard capsules after melting active ingredient in glycerin, polyethylene glycol chain, sesame oil, olive oil etc. The sheath may also be made with gelatin film to make a soft capsule.

[0016]

A liquid state formulation for oral dosage is produced using the active ingredient and melting sucrose, sorbitol, glycerin or other sweetener in water, to make an elixir or transparent syrup. Furthermore, by using an essential oil, with ethanol etc, it is possible to make an emulsion or suspension, by including gum arabic, traganth, polysorbate 80, [karubokishi mechiruseruro osunatoriumu] etc. These liquid state formulations may include desired flavoring, colorant, preservative etc

[0017]

An injectable form is produced with hydrochloric acid, sodium hydroxide, lactic acid, sodium lactate, sodium hydrogen phosphate **, or with sodium dihydrogen phosphate ** or with other pH adjustment agent, sodium chloride, fructose or other isotonic agent, which melts the active ingredient in injectable distilled water, produced using sterile filters, and filled ampoules, or by lyophilizing under vacuum. Furthermore, by using mannitol, dextrin, cyclodextrin, gelatin, etc it is possible to produce an injectable form of soluble type. An active ingredient emulsifying agent may include lecithin, polysorbate 80, polyoxyethylene hardening ** to make an injectable emulsion.

[0018]

A rectal administration agent is produced by humidifying active ingredient and cacao butter, aliphatic acid and base for triglyceride, polyethylene glycol chain or other suppository sink that is packed in type and cools, or after melting active ingredient in polyethylene glycol chain, soybean oil etc, with a sheath of gelatin film.

[0019]

An external skin preparation is produced by adding active ingredient to the white vaseline, beeswax, liquid paraffin, polyethylene glycol chain etc, humidifying or kneading the combination to make an ointment, or rosin, using alkyl acrylate ester polymer or other adhesive and kneading the combination after spreading/displaying or extending with a polyethylene or other nonwoven fabric.

[0020]

An inhalant is produced with active ingredient in freon gas or other propellant melting or dispersing, or filled in a pressure resistant vessel as an aerosol agent.

[0021]

A dose of peptide of this invention differs depending upon age, body weight and disease of patient (cattle, sheep or other animal including) of transmissible spongiform encephalopathy, but usually with per day approximately 1 to 500 mg, divided into one or several dosage times, as desirable to prescribe.

[0022]

Below, the invention is explained more concretely, on the basis of a Working Example, but not limited to this. In addition, protein which has amino acid sequence of synthetic peptide which applies this activity measurement method to the total length of an amino acid sequence of all kinds of prion protein, possesses chaperone activity according to the present invention, making use of known amino acid characteristics or other taxonomy and (40% or higher) homology, the claims of this invention include utilizing an amino acid sequence entirely or portion thereof to include an amino acid sequence where homology is high, for the same objectives (i.e., treatment, or prevention of transmissible spongiform encephalopathy or development of diagnostics). Furthermore, as for this activity measurement method, as for being able to utilize the method also for, prevention of transmissible spongiform encephalopathy or research method for a drug or diagnostic, it is not something where application method is limited in the this working example.

[0023]

[Working Example (s)]

First, various synthetic peptides were prepared.

Synthetic peptides used for this working example are shown in the below-mentioned Table 1.

[0024] [Table 1]

	SEQUENCE
1	GIGKFLKKAKKFAKAFVKILKK-CONH ₂
2	LAKLLKALAKLLKK-CONH ₂
3	KTNMKHMAGAAAAGAVVGGLG-COOH
4	GLGGVVAGAAAAGAMHKMNTK-COOH
5	DAPAAPAGPAVPV-COOH
6	VPVAPGAPAAPAD-COOH
7	ISRGSGRTWSNRY-COOH
8	ISDGSGDTWSNDY-COOH
9	CH ₃ CONH-KTNMKHMAGAAAAGAVVGGLG-COOH

[0025]

As described in the publications of prior art [A. Iwahori et al., Biol Pharm Bull. 20(3):267-70 (1997); K. Johnsson et al., Nature 365(6446):530-2. (1993); G. Forloni et al., Nature 362(6420):543-6 (1993); C. Soto et al., Lancet 355(9199):192-7 (2000)] the amino acid sequences in Sequence Numbers 1, 2, 3 and 5 are public knowledge. Amino acid sequences in Sequence Numbers 4, 6, 7, 8 and 9 are included in claims of this invention. In addition, for amino acid sequence which are stated in Sequence Number 1,2,3,4 and 9, at least one amphipathic amino acid sequence may be included, such as exists in calcitonin, with basic amino acid lysine residues or other amino acid.

[0026]

Next, decarboxylase activity measurement was done. This measurement method is as follows. 2.98 mM oxalo acetate (1.7 ml ; 50mM MOPS, 0.15M NaCl, pH 7.5) with trifluoro-ethanol (TFE) (0.2 ml) by addition to a spectroscopy cell, at room temperature, and with 5 min agitation. Adding 0.1 ml of measured sample of 2.0 mM concentration to solution of after stirring,, to obtain a reaction solution total amount of 2 ml, at room temperature, with 1 minute agitation (Iuchi, HS-3B, rotation speed 4). After that, churning was stopped, absorbance at 285 nm was measured with using a spectroscopy (UltraSpec3100pro, Amersham Biosciences corp.). A sample of Sequence Number 2 (0.2 mM) was used as control sample.

[0027]

After starting the reaction, absorbance was reduced for an amino acid sequence as stated in Sequence Number 2, after 2500 seconds. Similarly, absorbance was reduced for oxalo-acetate using amino acid Sequence Number 2. Concerning test peptide (Sequence Number 1, 3, 4, 5, 6, 7 and 8) absorbance was reduced similarly, with the relative ratio activity for amino acid sequences in Sequence Number 2 of test peptide with below-mentioned Mathematical Formula 1.

[0028]

[Mathematical Formula 1]

$$[\text{-----}] = (c-b) / (a-b)$$

[0029]

This measurement result is shown in Table 2

[0030]

[Table 2]

*****	*****
1	0.32
2	1.00
3	0.12
4	0.12
5	0.02
6	0.04
7	NT
8	NT
9	NT

[0031]

As for amino acid sequences stated in Sequence Number 3 and 4, from TFE existing at the start of the reaction, it became clear from this result that these sequences possessed decarboxylation activity.

[0032]

Next, chaperone activity measurement of synthetic peptide was done. This measurement method is as follows. 2.98 mM oxalo-acetate (1.7 ml ; 50mM MOPS, 0.1 5M NaCl, pH 7.5) with TFE (0.2 ml) added to a spectroscopy cell, at room temperature, with agitation for 5 min, adding 0.1 ml to the measurement sample of 2.0 mM concentration to solution of after stirring,, to obtain a reaction solution total amount as 2 ml, at room temperature, with 1 minute agitation (Iuchi, HS-3B, rotation speed 4). After that, churning was stopped, and absorbance at 285 nm was measured with a spectroscopy (Ultroaspec 3100pro, Amersham Biosciencescorp.). Amino acid sequence (0.2 mM) stated in 2.0 mM Sequence Number 3 (or 4) was used as a control sample. Absorbance was reduced after starting the reaction for Sequence Number 3, after 2500 seconds. Similarly, absorbance was reduced for oxalo-acetate using Sequence Number 3.

[0033]

Next, a sample of 0.2 ml test peptide (Sequence Number 5, 6 ,7 or 8) was added in place of TFE of the above-mentioned operation, and agitated for 5 min at room temperature. Then , adding 0.1 ml of sample (Sequence Number 3) of 2.0 mM concentration to solution of after stirring ,to obtain a reaction solution total amount of 2 ml, at room temperature, with 1 minute of agitation (Iuchi, HS-3B, rotation speed 4). After stopping churning, absorbance at 285 nm was measured with the spectroscopy. Relative ratio activity for amino acid sequence which is stated in Sequence Number 3 (or 4) of test peptide with below-mentioned Mathematical Formula 2 was sought.

[0034]

[Mathematical Formula 2]

$$[\text{-----}] = (f-e) / (d-e)$$

[0035]

this measurement result is shown in Table 3 .

[0036]

[Table 3]

試料	相対比活性
配列番号 3 (200 μ M) + TFE	1.00
配列番号 4 (200 μ M) + TFE	1.00
配列番号 3 (200 μ M) + 配列番号 5 (100 μ M)	0.12
配列番号 3 (200 μ M) + 配列番号 5 (200 μ M)	0.38
配列番号 3 (200 μ M) + 配列番号 6 (100 μ M)	0.42
配列番号 3 (200 μ M) + 配列番号 6 (200 μ M)	0.44
配列番号 3 (200 μ M) + 配列番号 7 (100 μ M)	NT
配列番号 3 (200 μ M) + 配列番号 7 (200 μ M)	NT
配列番号 3 (200 μ M) + 配列番号 8 (100 μ M)	NT
配列番号 3 (200 μ M) + 配列番号 8 (200 μ M)	NT

[0037]

From this result, as for peptide (Sequence Number 6) of this invention, it became clear to have possessed chaperone activity.

[0038]

Furthermore, chaperone activity measurement of a synthetic peptide was done with another method. This measurement method is as follows. In spectroscopy cell, 2 mM Sequence Number 3 (or 4) (0.2 ml), in mixed solution of buffer (50 mM MOPS, 0.1 5M NaCl, pH 7.0) (1.4 ml), was agitated for 48 hours (Iuchi, HS-3B, rotation speed 4) at room temperature including 2 mM measurement sample (Sequence Number 5, 6, 7, 8) (0.2 ml). A total amount of 2.0 ml was obtained after stirring, adding 2.98mM oxalo-acetate (0.2 ml), and then furthermore with 1 minute agitation. Churning was stopped, and absorbance measured at 285 nm at 5000 second after the starting the reaction, using a spectroscopy (Ultraspec3100pro, Amersham Biosciences Corp.). As a control sample, absorbance measured with Sequence Number 3 with trifluoro-ethanol (TFE) existing was designated as 1.00.

[0039]

This measurement result is shown in Table 4.

[0040]

[Table 4]

試料	相対比活性
配列番号 3 (200 μ M) + TFE (+)	1.00
配列番号 3 (200 μ M) + TFE (-)	0.59
配列番号 4 (200 μ M) + TFE (+)	0.70
配列番号 4 (200 μ M) + TFE (-)	0.54
配列番号 3 (200 μ M) + 配列番号 5 (200 μ M)	0.62
配列番号 3 (200 μ M) + 配列番号 6 (200 μ M)	0.60
配列番号 3 (200 μ M) + 配列番号 7 (200 μ M)	0.65
配列番号 3 (200 μ M) + 配列番号 8 (200 μ M)	0.57

[0041]

From this result, as for peptide (Sequence Number 6 and 7) of this invention it became clear to have possessed chaperone activity.

[0042]

Next, decarboxylation activity measurement was done using another different method. This measurement method is as follows. In a spectroscopy cell, 2 mM Sequence Number 9 (0.2 ml), in mixed solution of buffer (50 mM MOPS, 0.1 M NaCl, pH 7.0) (1.4 ml), is agitated for 48 hours (Iuchi, HS-3B, rotation speed < 4) with room temperature including TFE (0.2 ml). An obtained total amount of 2.0 ml including, after stirring, 2.98 mM oxaloacetate (0.2 ml), is furthermore agitated for 1 minute. Churning is stopped, and absorbance after 5000 seconds measured with a spectroscopy (UltraspecS 100pro, Amersham Biosciences Corp). As control sample, absorbance measured with Sequence Number 2 from trifluoro-ethanol (TFE) existing was designated as 1.00.

[0043]

This measurement result is shown in Table 5.

[0044]

[Table 5]

*****	****	*****
**** 2 200 μ M, + TFE (+)	0.195	1.00
**** 2 200 μ M + TFE (-)	0.159	
**** 9 200 μ M + TFE (+)	0.169	0.61
**** 9 200 μ M + TFE (-)	0.147	

[0045]

From this result, as for amino acid sequence which is stated in Sequence Number 9 under TFE existing it became clear to have possessed decarboxylation activity.

[0046]

Next, chaperone activity measurement of chlorpromazine, which is used as antipsychotic drug, was done. This measurement method is as follows. In a spectroscopy cell, 2 mM Sequence Number 9 (0.2 ml), in mixed solution of buffer (50 mM MOPS, 0.1 M NaCl, pH 6.0) (1.4 ml), was agitated for 48 hours (Iuchi, HS-3B, rotation speed 4) at room temperature, including 2 mM chlorpromazine. A total amount of 2.0 ml after stirring, adding 2.98 mM oxaloacetate (0.2 ml), was furthermore agitated for 1 minute. Churning was stopped, absorbance measured at 285 nm after 5000 seconds after starting the reaction with a spectroscope (Ultraspec3100pro, Amersham Biosciences Corp.) As control sample, absorbance measured for Sequence Number 9 with chlorpromazine existing was designated as 1.00.

[0047]

This measurement result is shown in Table 6.

[0048]

[Table 6]

試料	相対比活性
クロルプロマジン (200 μ M) (pH6.0)	1.00
緩衝溶液中でのオキサロアセートの自己分解 (pH6.0)	1.00
配列番号9 (200 μ M) +クロルプロマジン (200 μ M) (pH6.0)	1.31

[0049]

From this result, as for chaperone activity measurement method of this invention it became clear to be able to search low molecular weight organic compound as treatment drug for transmissible spongiform encephalopathy.

[0050]

[Effects of the Invention]

Above-mentioned peptide has molecular chaperone activity, and possesses application for treatment, prevention or diagnostic for transmissible spongiform encephalopathy.

[0051] [Sequence]

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<120> Synthetic Peptide with Chaperone Activity

<130> IY04651

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IP 2002-200884 2002-7-10

IP 2002-268260 2002-9-13

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 Lys Met Asn Thr Lys
 20

Drawings

Molecular determinants of the physicochemical properties of a critical prion protein region comprising residues 106–126

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Prion diseases are marked by the cerebral accumulation of conformationally modified forms of the cellular prion protein (PrP^C), known as PrP^{res}. The region comprising the residues 106–126 of human PrP seems to have a key role in this conformational conversion, because a synthetic peptide homologous with this sequence (PrP106–126) adopts different secondary structures in different environments. To investigate the molecular determinants of the physicochemical characteristics of PrP106–126, we synthesized a series of analogues including PrP106–126 H_D, PrP106–126 A and PrP106–126 K, with L-His → D-His, His → Ala and His → Lys substitutions respectively at position 111, PrP106–126 NH₂ with amidation of the C-terminus, PrP106–126 V with an Ala → Val substitution at position 117, and PrP106–126 VNH₂ with an Ala → Val substitution at position 117 and amidation of the C-terminus. The analysis of the secondary structure and aggregation properties of PrP106–126 and its analogues showed the following. (1) His¹¹¹ is central to the conformational changes of PrP peptides. (2)

Amidation of the C-terminal Gly¹²⁶ yields a predominantly random coil structure, abolishes the molecular polymorphism and decreases the propensity of PrP106–126 to generate amyloid fibrils. (3) PrP106–126 V, carrying an Ala → Val substitution at position 117, does not demonstrate a fibrillogenic ability superior to that of PrP106–126. However, the presence of Val at position 117 increases the aggregation properties of the amidated peptide. (4) Amyloid fibrils are not required for neurotoxicity because the effects of PrP106–126 NH₂ on primary neuronal cultures were similar to those of the wild-type sequence. Conversely, astroglial proliferation is related to the presence of amyloid fibrils, suggesting that astrogliosis in prion encephalopathies without amyloid deposits is a mediated effect rather than a direct effect of disease-specific PrP isoforms.

Key words: amyloid, prion protein peptides, secondary structure modifications.

INTRODUCTION

Prion diseases such as scrapie of sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt–Jakob disease ('CJD') and Gerstmann–Sträussler–Scheinker disease (GSS) of humans are characterized by the accumulation of abnormal forms of the cellular prion protein (PrP^C), termed PrP^{res}, in the brain [1]. In contrast with PrP^C, PrP^{res} is partly resistant to digestion with protease and has a marked tendency to form insoluble aggregates and amyloid fibrils [2–4]. The accumulation of PrP^{res} and PrP amyloid in the brain is thought to be responsible for the nerve cell degeneration, astrogliosis and activation of microglial cells observed in prion-related encephalopathies [5–7].

NMR studies of recombinant murine PrP indicate that the normal protein is composed of two structurally distinct moieties: an extended N-terminal segment (residues 23–125) with features of a flexibly disordered polypeptide chain, and a well-defined globular domain (residues 126–231) with three α -helices and a two-stranded anti-parallel β -sheet [8–10]. The transition from PrP^C to PrP^{res} involves a striking conformational change with a decrease in α -helical secondary structure (from 42% to 30%) and a remarkable increase in β -sheet content (from 3% to 43%) [11,12]. This rearrangement is accompanied by the acquisition of

abnormal physicochemical properties, including insolubility in non-denaturing detergents and partial resistance to digestion with proteinase K.

Previous studies have shown that a synthetic peptide homologous with residues 106–126 of human PrP (PrP106–126) exhibits some of the pathogenic and physicochemical properties of PrP^{res} [5–7,13–15]. Like PrP^{res}, this peptide causes nerve cell death by apoptosis and induces hypertrophy and proliferation of astrocytes and activation of microglial cells *in vitro* [5–7]. It is noteworthy that the neurotoxicity of the peptide requires the expression of endogenous PrP, which is consistent with the observation that neuronal death in scrapie infection *in vivo* is dependent on PrP^C synthesis [16,17]. It also increases the membrane microviscosity of a variety of cells, including neurons and astrocytes [18,19]. PrP106–126 shows a remarkable conformational polymorphism, acquiring different secondary structures in different environments [15]; nevertheless, it tends to adopt a β -sheet conformation in buffered solutions and aggregates into amyloid fibrils that are partly resistant to digestion with protease. These data indicate that the PrP region including residues 106–126 might be the nido at which conformational change is initiated in the conversion of PrP^C to PrP^{res}. This view is supported by the observation that the N-terminal half of PrP

Abbreviations used: GSS, Gerstmann–Sträussler–Scheinker; PrP, prion protein; PrP^C, cellular PrP; PrP^{res}, pathological and protease-resistant isoform of PrP; PrP106–126, synthetic peptide comprising residues 106–126 of human PrP; PrP106–126 H_D, with L-His → D-His substitution at position 111; PrP106–126 A, with His → Ala substitution at position 111; PrP106–126 K, with His → Lys substitution at position 111; PrP106–126 V, with Ala → Val substitution at position 117; PrP106–126 NH₂, with amidation at the C-terminus; PrP106–126 VNH₂, with Ala → Val substitution at position 117 and amidation of the C-terminus; TFE, 2,2,2-trifluoroethanol.

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(residues 23–125) is highly flexible and more susceptible to structural transitions than the C-terminal globular domain, and that the deletion of residues 23–88 does not prevent the conversion of PrP^C to PrP^{Sc}, whereas the ablation of residues 108–121 or 122–140 together with residues 23–88 does so [20].

The present study was undertaken to investigate the molecular determinants of the conformational polymorphism of PrP106–126 and its propensity to form amyloid fibrils. Several peptide analogues containing amino acid changes were synthesized and their physicochemical properties were compared with the those of wild-type sequence. The selection of amino acid changes was based on the assumption that the conformational polymorphism and fibrillogenic properties of PrP106–126 depend at least in part on the presence of an ionizable residue (i.e. His¹¹¹) between the hydrophilic and hydrophobic domains of the peptide, and on electrostatic and hydrophobic interactions between molecules conferring stability to the β -sheet configuration. We also analysed the effects of the Ala \rightarrow Val substitution at position 117, a mutation linked to GSS disease. This effect was evaluated on the wild-type protein and the C-terminal amidated form of PrP106–126 (PrP106–126 NH₂). The latter was used because the amidation of the C-terminus greatly decreases the fibrillogenic properties of the peptide and allows the more effective detection of the influence of the amino acid substitution. Finally, we investigated how the physicochemical changes resulting from the amidation of the C-terminus and the Ala \rightarrow Val substitution at residue 117 affected the biological activity of the peptide.

EXPERIMENTAL

Peptide synthesis

The following peptides were used for the study: PrP106–126 (K¹⁰⁶TNMKHMAGAAAAGAVVGGLG; single-letter codes); PrP106–126 D₁₁₁, with an L-His \rightarrow D-His substitution at position 111; PrP106–126 A (K¹⁰⁶TNMKAMAGAAAAGAVVGGLG), with an His \rightarrow Ala substitution at position 111; PrP106–126 K (K¹⁰⁶TNMKKMAGAAAAGAVVGGLG), with a His \rightarrow Lys substitution at position 111; PrP106–126 V (K¹⁰⁶TNMKHMAGAAVAGAVVGGLG), with an Ala \rightarrow Val substitution at position 117; PrP106–126 NH₂ (K¹⁰⁶TNMKHMAGAAAAGAVVGGLG-NH₂), with amidation at the C-terminus; and PrP106–126 VNH₂ (K¹⁰⁶TNMKHMAGAAVAGAVVGGLG-NH₂), with an Ala \rightarrow Val substitution at position 117 and amidation at the C-terminus. The peptides were synthesized by solid-phase chemistry and purified by reverse-phase HPLC, as described previously [15]. Purity was always greater than 95%.

Preparation of peptide solutions

Peptides were dissolved in deionized water at 0.75 or 1.5 mg/ml (stock solutions). Under these conditions they were soluble, as deduced by the absence of a visible pellet after centrifugation at 13000 *g* for 10 min. The stock solutions were stable for 2 weeks at -80°C , as determined by reverse-phase HPLC (see below). Aliquots of stock solutions were added to 200 mM phosphate buffer, pH 5.0 or 7.0, to obtain final peptide concentrations of 0.25, 0.50, 0.75 or 1.0 mg/ml phosphate buffer. The actual concentration of each peptide in phosphate buffer was determined by HPLC analysis immediately after preparation of the samples and was used as the zero-time value in sedimentation experiments. To investigate how the α -helix-stabilizing solvent 2,2,2-trifluoroethanol (TFE) influenced the secondary structure, the peptides were first suspended in phosphate buffer, pH 5.0, at a con-

centration of 0.5 mg/ml; TFE was then added to the samples to a final concentration of 50% (v/v).

CD spectroscopy

The analysis was performed on 0.25 mg/ml peptide suspensions in phosphate buffer, pH 5.0 and 7.0, or phosphate buffer containing TFE, prepared as described above. The spectra were recorded after incubation for 1 h in quartz cells, with an optical path of 0.1 cm, by using a Jasco J-500 dichograph (Tokyo, Japan) at a scan speed of 2 nm/min. Mean residue ellipticities were calculated by using the following equation: $(\theta)_M = 3300AM/Cl$, where A is the observed dichroic absorbance, l is the path length in cm, C is the concentration of the peptide in g/l and M is the mean residue weight. The percentages of the secondary structure of the peptides were calculated by the method of Yang et al. [21].

Turbidity measurements

The analysis was performed on 0.25 mg/ml peptide suspensions in phosphate buffer, pH 5.0 and 7.0. The turbidity of the samples was determined immediately after their preparation (zero time) and after incubation for 24 h at room temperature, with a Perkin-Elmer Lambda 10 spectrophotometer at 600 nm.

Sedimentation experiments

The study was performed on 0.75 mg/ml peptide suspensions in phosphate buffer, pH 5.0. Aliquots of 20 μ l were incubated at 37°C for 0, 1, 2, 4 and 24 h, then chilled on ice and centrifuged at 13000 *g* for 10 min at 4°C . Supernatant (2 μ l) was injected into an HPLC apparatus equipped with a 3.9 mm \times 150 mm Delta-Pak C₁₈ column, a model P 4000 pump, a UV 2000 variable-wavelength detector, an AS 3000 autosampler (Waters, Milford, MA, U.S.A.) and an SP 4400 integrator operated in both peak height and area modes (Thermo Separations Products, Riviera Beach, FL, U.S.A.). MilliQ water/acetonitrile (75:25, v/v) containing 0.1% (v/v) trifluoroacetic acid was used as eluent, at a flow rate of 1 ml/min. The column eluates were monitored at 214 nm. The peptide concentrations in the supernatant at different times were expressed as percentages of the corresponding values determined at zero time.

Light and electron microscopy

Peptides were suspended in phosphate buffer, pH 5.0, at a final concentration of 1 mg/ml. After incubation for 24 h at 20°C , 50 μ l of each suspension was air-dried on gelatin-coated slides, stained with 1% (w/v) aqueous thioflavin S and analysed by fluorescence microscopy. For electron microscopy, 5 μ l of suspension was applied to Formvar-coated nickel grids, negatively stained with 5% (w/v) uranyl acetate and observed in an electron microscope (EM 109, Zeiss, Germany) at 80 kV.

Nerve cell cultures

Cerebral cortex was dissected from fetal rat brains on embryonic day 17. Cortical cells were dissociated and plated in 24-well dishes (3.5×10^5 cells per dish) at 37°C in humidified air/CO₂ (19:1), as described previously [5]; 24 h after being plated, the cultures were exposed to PrP106–126, PrP106–126 NH₂ or

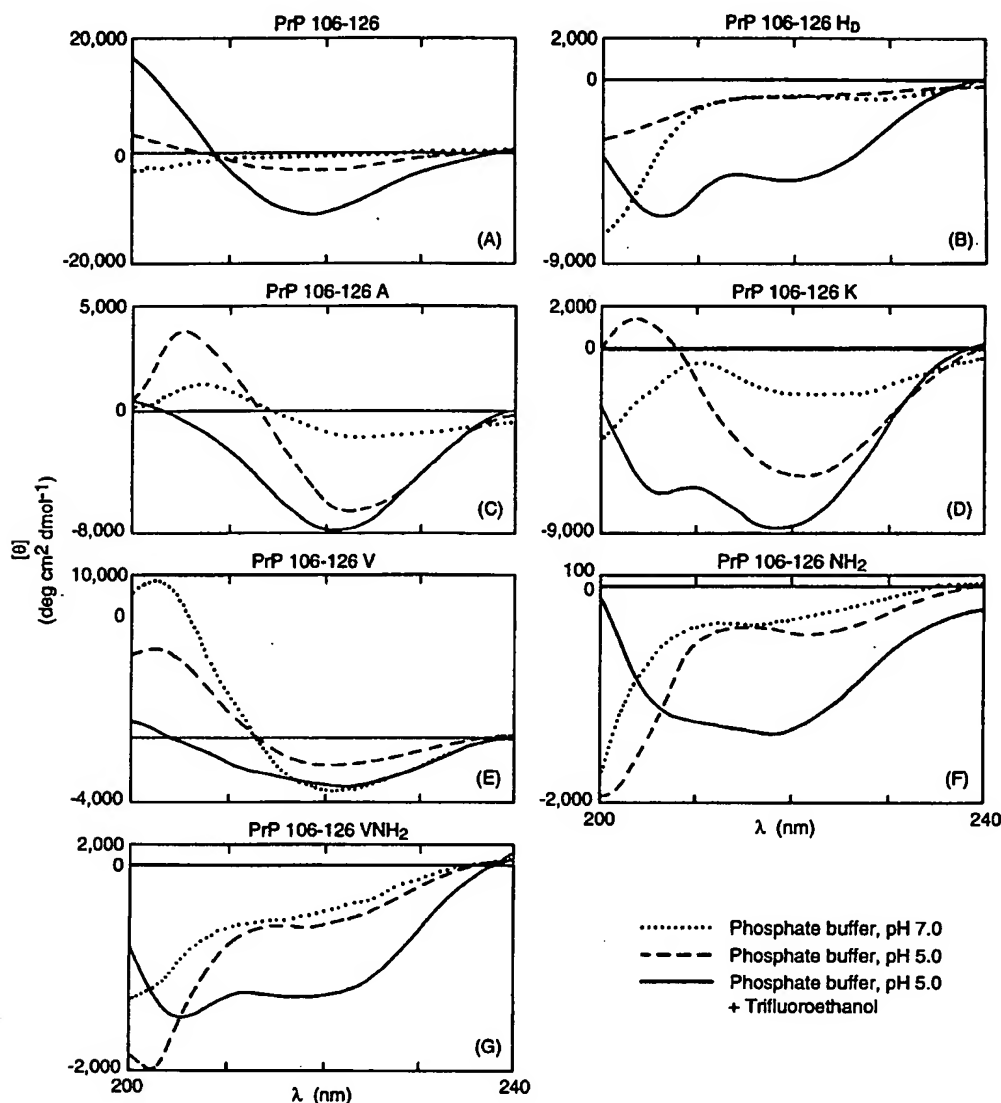


Figure 1 CD spectra of PrP106–126 and its analogues

PrP106–126 VNH₂ at 50 μ M for 7 days. Control cultures were exposed to vehicle only. Cell viability was then assessed by staining with Crystal Violet [0.5% in water/methanol (4:1)]. After washing, cells were dried and the staining intensity was determined densitometrically with an image analyser (IBAS 2.0; Zeiss, Cologne, Germany) [22].

Astroglial cultures

Glial cell cultures were prepared from newborn rat pups as described [6]. After 10 days of culture, flasks containing mixed glial cells were given fresh medium and shaken at 37 °C for 12–16 h. The supernatant containing microglia and oligodendrocytes was removed and replaced with fresh Dulbecco's modified Eagle's medium. The adherent cells (astrocytes) were then exposed for 5 min to 0.25% trypsin. After the addition of an equal volume of Dulbecco's modified Eagle's medium/10% (v/v) fetal calf serum, the cell suspension was centrifuged and the pellet was resuspended in medium containing 10% fetal calf

serum. Cells were plated at a density of 5×10^4 cells/ml [20] and exposed to PrP106–126, PrP106–126 NH₂ or PrP106–126 VNH₂ at 50 μ M for 7 days. Control cultures were exposed to vehicle only. Glial proliferation was assessed by staining with Crystal Violet and densitometric analysis [23,24].

RESULTS

Secondary structure of PrP peptides as deduced by CD spectroscopy

In a previous study we showed that PrP106–126 can adopt different conformations in different environments, e.g. a random coil structure in deionized water, a combination of random coil and β -sheet in 200 mM phosphate buffer, pH 7.0, a β -sheet conformation in phosphate buffer, pH 5.0, and an α -helical structure in TFE. A remarkable finding was the stability of the β -sheet secondary structure, which was not affected by the addition of TFE to a solution of the peptide in phosphate buffer, pH 5.0 [15].

Table 1 Percentages of secondary structure in peptide PrP106-126 and its analoguesResults are means \pm S.E.M. for at least three different experiments.

Row	Peptide	pH	TFE	Secondary structure (%)			Random coil
				α -Helix	β -Sheet	β -Turn	
A	PrP106-126	5.0	—	22 \pm 3	61 \pm 4	—	17 \pm 4
B		7.0	—	17 \pm 2	40 \pm 5	4 \pm 1	39 \pm 2
C		5.0	+	28 \pm 5	58 \pm 3	2 \pm 1	12 \pm 5
D	PrP106-126 H _D	5.0	—	5 \pm 1	12 \pm 5	5 \pm 3	78 \pm 7
E		7.0	—	4 \pm 2	4 \pm 3	11 \pm 5	81 \pm 9
F		5.0	+	48 \pm 6	16 \pm 3	5 \pm 2	31 \pm 7
G	PrP106-126 A	5.0	—	15 \pm 4	53 \pm 6	14 \pm 2	18 \pm 5
H		7.0	—	13 \pm 3	51 \pm 5	16 \pm 2	20 \pm 5
I		5.0	+	25 \pm 8	50 \pm 10	9 \pm 1	16 \pm 5
J	PrP106-126 K	5.0	—	22 \pm 3	51 \pm 6	12 \pm 3	15 \pm 2
K		7.0	—	9 \pm 2	24 \pm 5	18 \pm 2	51 \pm 7
L		5.0	+	40 \pm 8	42 \pm 10	2 \pm 1	16 \pm 5
M	PrP106-126 V	5.0	—	25 \pm 3	58 \pm 8	2 \pm 1	20 \pm 2
N		7.0	—	14 \pm 2	44 \pm 5	4 \pm 2	38 \pm 1
O		5.0	+	22 \pm 5	59 \pm 9	1 \pm 0	18 \pm 2
P	PrP106-126 NH ₂	5.0	—	5 \pm 2	11 \pm 5	11 \pm 7	73 \pm 13
Q		7.0	—	4 \pm 3	8 \pm 4	12 \pm 4	76 \pm 11
R		5.0	+	51 \pm 7	25 \pm 5	5 \pm 2	19 \pm 7
S	PrP106-126 VNH ₂	5.0	—	5 \pm 2	15 \pm 2	9 \pm 5	71 \pm 10
T		7.0	—	5 \pm 1	19 \pm 4	9 \pm 2	67 \pm 7
U		5.0	+	42 \pm 8	22 \pm 3	6 \pm 3	30 \pm 6

To unravel the molecular determinants of these structural properties, we generated PrP106-126 analogues with modifications of single amino acids (substitution of D-His, Ala or Lys for L-His¹¹¹; substitution of Val for Ala¹¹⁷; amidation of the C-terminus with or without the substitution of Val for Ala¹¹⁷) and analysed the effects of these changes on the conformational polymorphism of the peptides and the stability of the β -sheet conformation. Figure 1 and Table 1 show the CD spectra and percentages of secondary structure of PrP106-126 analogues in phosphate buffer, pH 5.0 and 7.0, and in phosphate buffer, pH 5.0, after the addition of TFE.

The characteristics of PrP106-126 were substantially altered by modifications of His¹¹¹. PrP106-126 H_D showed a striking decrease in β -sheet and α -helical content, with a parallel increase in random coil structure; this effect was observed at both pH 5.0 and 7.0 (Figure 1B, and Table 1, rows D and E). The addition of TFE to PrP106-126 H_D solutions in phosphate buffer markedly enhanced the α -helical content (Figure 1B, and Table 1, row F). PrP106-126 A showed a high proportion of β -sheet secondary structure at both pH 5.0 and 7.0, as indicated by a positive peak with a maximum at 206 nm and a broad negative band centred on 220 nm (Figure 1C, and Table 1, rows G and H); the only difference between the spectra recorded at neutral and acidic pH was the signal intensity, which was much stronger at pH 5.0. The addition of TFE to PrP106-126 A in phosphate buffer resulted in a slight increase in α -helix (Figure 1C, and Table 1, row I). PrP106-126 K adopted a predominantly β -sheet conformation in phosphate buffer at pH 5.0 (Figure 1D, and Table 1, row J); the β -sheet content decreased at pH 7.0 and the random coil became the prevalent secondary structure (Figure 1D, and Table 1, row K). The addition of TFE to PrP106-126 K in phosphate buffer caused an ordered arrangement with equal contributions of α -helix, as deduced by two negative bands at 206 and 220 nm, and

Table 2 Turbidity measurements on PrP106-126 and its analogues

Peptides were suspended in 200 mM phosphate buffer, pH 5.0 or 7.0, at a concentration of 0.25 mg/ml; and the difference in D_{600} between zero time and after incubation for 24 h at room temperature was calculated. Results are means \pm S.E.M. for at least four determinations. * $P < 0.05$ compared with the peptide suspension at pH 5.0 (Student's t test).

Peptide	pH...	ΔD_{600} between 0 and 24 h of incubation	
		5.0	7.0
PrP106-126		0.085 \pm 0.008	0.114 \pm 0.032*
PrP106-126 H _D		0.071 \pm 0.010	0.078 \pm 0.014
PrP106-126 A		0.121 \pm 0.005	0.126 \pm 0.002
PrP106-126 K		0.075 \pm 0.010	0.080 \pm 0.011
PrP106-126 V		0.081 \pm 0.001	0.110 \pm 0.001*
PrP106-126 NH ₂		0.064 \pm 0.003	0.066 \pm 0.003
PrP106-126 VNH ₂		0.069 \pm 0.002	0.096 \pm 0.011*

β -sheet, as indicated by a more intense, broad negative absorption at 220 nm (Figure 1D, and Table 1, row L).

The substitution of Val for Ala¹¹⁷ (PrP106-126 V) had no significant effect on the spectral features and structural stability of PrP106-126, which had a high β -sheet content in phosphate buffer (Figure 1E, and Table 1, rows M and N) and also in the presence of TFE (Figure 1E, and Table 1, row O). Amidation of the C-terminal Gly¹²⁶ (PrP106-126 NH₂) decreased the propensity to adopt the β -sheet structure in phosphate buffer, at both pH 5.0 and 7.0; under these conditions, the peptide showed a random coil arrangement, as indicated by the strong negative band at 200 nm (Figure 1F, and Table 1, rows P and Q). The addition of TFE to PrP106-126 NH₂ in phosphate buffer, pH 5.0, caused a striking increase in α -helical structure (Figure 1F, and Table 1, row R). The substitution of Val for Ala¹¹⁷ of the amidated peptide (PrP106-126 VNH₂) slightly changed the spectral features in phosphate buffer, at both pH 5.0 and 7.0; this change consisted of an increased intensity of the negative band at 220 nm, indicating a higher degree of β -sheet secondary structure (Figure 1G, and Table 1, rows S and T). The addition of TFE to PrP106-126 VNH₂ in phosphate buffer, pH 5.0, resulted in an increased α -helical content (Figure 1G, and Table 1, row U); this increase was less pronounced than that observed for PrP106-126 NH₂.

Aggregation properties of PrP106-126 peptides

The ability of PrP106-126 analogues to form macroaggregates was first assessed by turbidity measurements. The peptides were suspended in phosphate buffer, pH 5.0 or 7.0, at 0.25 mg/ml, and the difference in absorbance between zero time and after incubation for 24 h at room temperature was calculated (Table 2). At zero time, no significant differences in optical density were observed between the different peptide suspensions at both pH 5.0 and 7.0; in a typical experiment at pH 5.0, the following initial values (means \pm S.E.M. for four replicates) were recorded: PrP106-126, 0.058 \pm 0.005; PrP106-126 H_D, 0.055 \pm 0.004; PrP106-126 A, 0.065 \pm 0.008; PrP106-126 K, 0.055 \pm 0.09; PrP106-126 V, 0.062 \pm 0.004; PrP106-126 NH₂, 0.051 \pm 0.008; PrP106-126 VNH₂, 0.053 \pm 0.009. After incubation for 24 h, PrP106-126 suspensions showed an increase in turbidity values that was higher at pH 7.0 than at pH 5.0. Similar figures were obtained with PrP106-126 V and PrP106-126 VNH₂, sug-

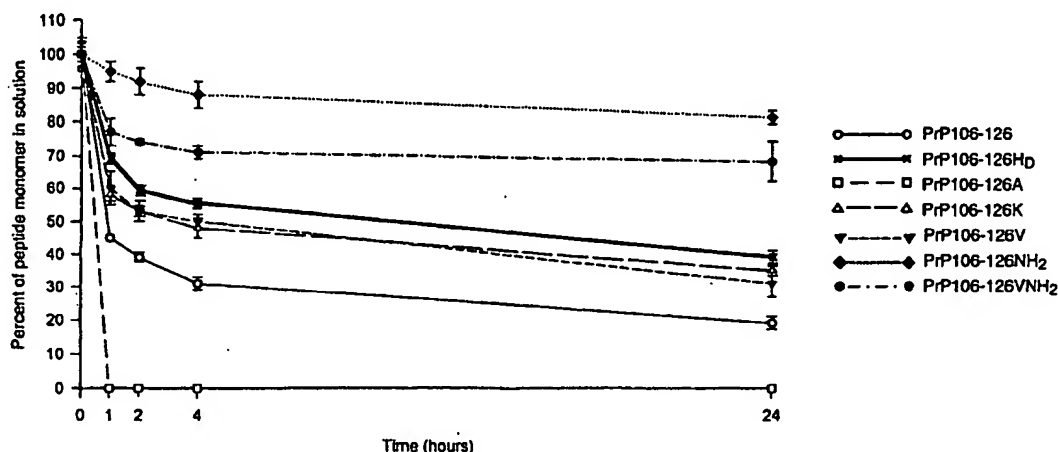


Figure 2 Sedimentation pattern of PrP106–126 and its analogues

Each point represents the percentage of peptide monomer in the 13000 *g* supernatant fraction of peptide solutions in 200 mM phosphate buffer, pH 5.0, incubated at room temperature for different durations. Results are means \pm S.E.M. for three determinations.

gesting that the neutral pH and the amino acid change linked to GSS disease enhance these peptides' ability to aggregate. In contrast, the increases in turbidity of the analogues carrying the substitution of D-His, Ala or Lys for L-His¹¹¹ or the amidation of Gly¹²⁶ were similar at pH 5.0 and 7.0; however, the magnitude of the increase differed between these peptides, being highest in PrP106–126 A and lowest in PrP106–126 NH₂. With regard to PrP106–126 A, the increase in turbidity occurred very rapidly and reached a plateau within 1 h (results not shown), suggesting that this peptide is highly insoluble in buffered solutions. It is noteworthy that the decrease in aggregation capacity and the sensitivity to pH variations of PrP106–126 NH₂ were partly restored by the substitution of Val for Ala¹¹⁷.

These results were consistent with those obtained by HPLC determination of the proportion of the peptides that was not precipitated by centrifugation. Whereas turbidimetry revealed the aggregated fraction, sedimentation enabled us to quantify the soluble fraction. This was lowest for PrP106–126 A, followed by PrP106–126, PrP106–126 V, PrP106–126 K, PrP106–126 H_D, PrP106–126 VNH₂ and PrP106–126 NH₂. All peptides showed a decrease in soluble fraction with time; the decrease was extremely rapid for PrP106–126 A, which was already undetectable in the supernatant after 1 h owing to its high insolubility (Figure 2).

Ultrastructural and staining properties of PrP peptide assemblies

The nature of the aggregates generated by PrP106–126 and its analogues was determined by electron microscopy after negative staining and by fluorescence microscopy after treatment with thioflavin S. As reported previously, PrP106–126 formed dense meshes of straight, unbranched fibrils 4–8 nm in diameter and up to 2.0 μ m long (Figure 3A). The fibrillar assemblies showed yellow fluorescence after staining with thioflavin S. Similar findings were observed with peptides with the substitution of Lys for His¹¹¹ (results not shown) and of Val for Ala¹¹⁷ (Figure 3D). Conversely, PrP106–126 H_D showed a striking decrease in density, length and diameter of the fibrils (Figure 3B).

The most remarkable change in the fibrillogenic properties of the peptide was seen with PrP106–126 A, of which the aggregates consisted primarily of amorphous material and, to a smaller

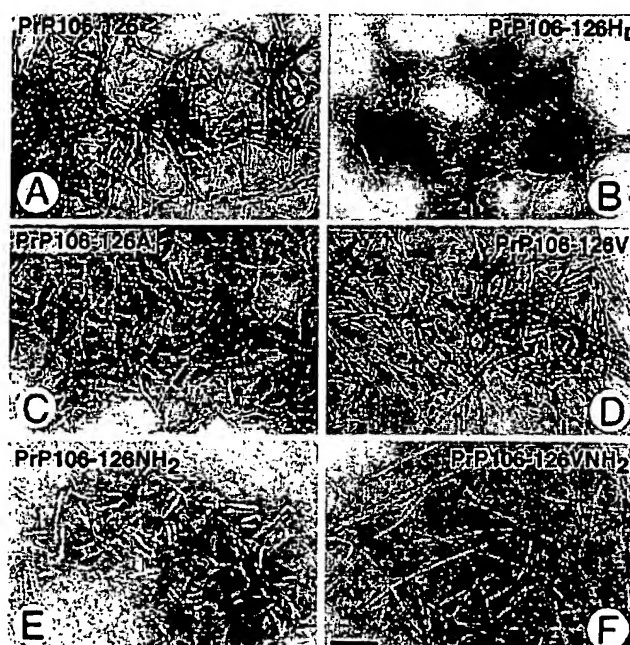


Figure 3 Electron micrographs of fibrils generated *in vitro* by PrP106–126 and its analogues

Shown are PrP106–126 (A), PrP106–126 H_D (B), PrP106–126 A (C), PrP106–126 V (D), PrP106–126 NH₂ (E) and PrP106–126 VNH₂ (F). Scale bar, 100 nm.

extent, of thin, short filamentous structures with an irregular profile (Figure 3C), lacking the staining properties of amyloid. PrP106–126 NH₂ also had a lower fibrillogenic ability than PrP106–126; this was partly restored in PrP106–126 VNH₂, whereas PrP106–126 NH₂ generated a few straight fibrils 4–8 nm in diameter and less than 0.2 μ m in length (Figure 3E). The morphology and staining properties of the peptide assemblies formed by PrP106–126 VNH₂ were similar to those of PrP106–

Table 3 Effects of PrP106–126 analogues on nerve and astroglial cells

Cultures of neurons and astrocytes were exposed for 7 days to the peptides [5,6]. Neuronal viability and astroglial proliferation were estimated by a colorimetric method. Results are expressed as percentage of control and are means \pm S.E.M. for six to twelve determinations. * $P < 0.01$ compared with the relevant control group (Dunnett's test).

Peptide	Nerve cell viability		Astroglial proliferation	
	Control	Peptide	Control	Peptide
PrP106–126	100 \pm 5.7	23.6 \pm 5.8*	100 \pm 3.0	265 \pm 10*
PrP106–126 NH ₂	100 \pm 6.2	23.0 \pm 7.9*	100 \pm 6.0	119 \pm 4.0
PrP106–126 VNH ₂	100 \pm 4.8	39.9 \pm 9.1*	100 \pm 10	164 \pm 2.0*

126 (Figure 3F); however, the fibril density was significantly lower than for the native sequence.

Biological activities of PrP106–126 analogues with different aggregation properties

Previous studies showed that the highly fibrillogenic PrP106–126 is neurotoxic and induces astroglial proliferation *in vitro*. To investigate whether these biological properties are related to the aggregation state of the peptide, primary cultures of neurons and astrocytes were exposed to analogues with diverse abilities to assemble into amyloid-like fibrils. In particular, PrP106–126 was compared with the poorly fibrillogenic PrP106–126 NH₂ and with PrP106–126 VNH₂, which had an intermediate propensity to aggregate. The study showed that the neurotoxicity of PrP106–126 analogues was not dependent on their fibrillogenic properties because all peptides induced a similar decrease in nerve cell viability after 7 days of treatment at 50 μ M. Conversely, the effects on astrocytes seemed to be related to the aggregation state because PrP106–126 NH₂ was unable to induce astroglial proliferation, whereas PrP106–126 VNH₂ showed partial activity (Table 3).

DISCUSSION

The sequence comprising residues 106–126 of human PrP corresponds to a highly conserved region of the molecule, located in the flexibly disordered N-terminal segment adjacent to the structurally well-defined globular domain [9,10]. This region is required for the conversion of PrP^C into PrP^{Sc} [20]. Studies *in vitro* suggest that it might have a central role in the conformational change of PrP and in the pathogenesis of tissue changes associated with the accumulation of PrP^{Sc} in the brain [5–7,12–17].

A recent study [25] on recombinant PrP corresponding to human residues 91–231 showed that reduction of the disulphide bond between Cys¹⁷⁹ and Cys²¹⁴ resulted in a conformational change from a predominantly α -helical to a β -sheet structure. Most of this rearrangement seemed to occur within the C-terminal domain, whereas residues 91–126 were found to be unstructured in both the α -helical and β -sheet forms of the protein. The discrepancy between this observation and our results could be due to profound differences in structure and behaviour when the PrP region comprising residues 106–126 is isolated as a peptide or is a part of the PrP polypeptide chain. However, previous studies showed that the disulphide bond is required for PrP^{Sc} formation [20]; thus the change from α -helical to β -sheet structure after reduction of the protein might be

different from that occurring *in vivo*. In this regard, it is noteworthy that the protease-resistant core of PrP^{Sc} corresponds approximately to residues 90–231 and includes the segment 90–125. Because this segment is flexible and protease-sensitive in PrP^C, it must undergo a significant conformational change in the conversion to PrP^{Sc} that precludes its accessibility to proteases.

We have previously shown that the synthetic peptide PrP106–126 adopts different conformations in different environments, although it has a high propensity to form stable β -sheet structures and to assemble into amyloid fibrils [13–15]. Because the secondary structure of the peptide is markedly influenced by pH, its physicochemical properties might be at least partly related to His¹¹¹, which is the only residue that changes in ionization state between pH 5.0 and 7.0 and is located in a critical region between the N-terminal polar head (KTNMKH) and the long hydrophobic tail (MAGAAAAGAVVGGGLG). The relevance of His¹¹¹ to the conformational change of PrP and the pathogenesis of prion diseases is suggested by the observations that (1) human PrP^C undergoes proteolytic cleavage between Lys¹¹⁰ and His¹¹¹, whereas the abnormal PrP species associated with prion diseases do not [26], and (2) transgenic mice expressing Syrian hamster PrP carrying the double substitution of the more hydrophobic residue Ile for Lys¹¹⁰ and His¹¹¹ spontaneously develop spongiform changes and astrogliosis of the grey matter, similar to those observed in scrapie-infected mice [27].

The present study further supports the view that His¹¹¹ is a critical site of the protein. The substitution of D-His¹¹¹ for L-His¹¹¹ abolished the pH-dependent conformational polymorphism of PrP106–126, caused a loss of stability of the β -sheet structure and decreased the peptide's fibrillogenic ability. Conceivably these effects are related to the fact that the orientation of the imidazole moiety of D-His is opposite to that of L-His; this might cause steric hindrance with neighbouring Lys¹¹⁰ and Met¹¹² residues, inducing the formation of a random coil structure. In contrast, the substitution of the highly hydrophobic, non-ionizable residue Ala for His¹¹¹ caused the peptide to become insoluble in buffered solutions. Although PrP106–126 A showed a high proportion of β -sheet secondary structure, it formed mainly amorphous aggregates. This is most probably due to the prevalence of the intramolecular hydrophobic forces of the lipophilic residues on the kinetics of fibril assembly. Unexpectedly, replacing His¹¹¹ with Lys (a polar residue that is always ionized in the pH range 5.0–7.0) modified the pH-dependent structural polymorphism of the peptide, although it did not affect its ability to form amyloid fibrils. As mentioned above, His¹¹¹ is located between the hydrophobic and hydrophilic portions of PrP106–126; this molecular environment might affect the pK_a of Lys at this site. Furthermore, the β -sheet secondary structure of PrP106–126 K was largely maintained in TFE, suggesting that its stability depends on an ionized residue at position 111.

Our results support previous observations that the β -sheet content is a key factor for amyloid fibril formation. The only exception was the substitution of Ala for His¹¹¹, as discussed above. Conversely, there was no simple and direct relation between the amount of β -sheet secondary structure of PrP106–126 analogues and their aggregation properties, as deduced by CD spectroscopy and turbidimetry respectively. The aggregation abilities of PrP106–126 and PrP106–126 V were greater at pH 7.0 than pH 5.0, despite both peptides' having a higher content of β -sheet structure at acidic pH. Furthermore, the quantity of aggregates of PrP106–126 K was similar at pH 5.0 and 7.0, although the β -sheet content was distinctly larger at pH 5.0. These apparent inconsistencies are due to the fact that CD spectroscopy and turbidimetry explore peptide populations in

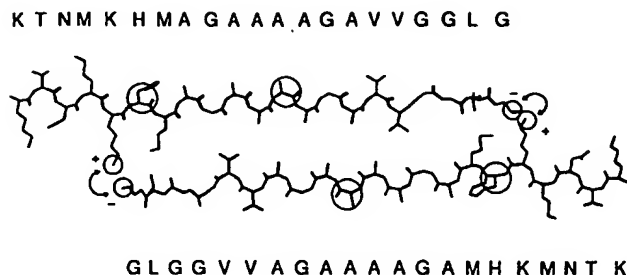


Figure 4 Proposed model of β -sheet formation by PrP106–126, showing an antiparallel alignment of two molecules of PrP106–126

The two ends of the secondary β -sheet structure are stabilized by ion-pair interactions. The residues His¹¹¹, Val¹¹⁷, the N-terminal Lys¹⁰⁶ and C-terminal Gly¹²⁶ are highlighted.

different physical states (the former detects the soluble fraction, whereas the latter reveals insoluble macroaggregates), so a correlation between the two might not always be apparent. PrP106–126 and PrP106–126 V are more soluble at pH 5.0 than at pH 7.0 owing to the protonation of His¹¹¹, which decreases the propensity of the peptides to aggregate by antagonizing the intramolecular hydrophobic forces of the lipophilic core. This allows the presence of a larger population of monomers or oligomers in solution that are capable of adopting a β -sheet secondary structure. With PrP106–126 K, His¹¹¹ was replaced by a residue that is not sensitive to pH change; aggregation was therefore not affected.

The contribution of the C-terminal carboxy group to the physicochemical properties of amyloid peptides has been investigated by Terzi et al. [28,29], by using the fragment 25–35 of the β -protein involved in the pathogenesis of Alzheimer disease. Removal of the C-terminal electric charge by amidation led to a predominantly random-coil structure and inhibited fibril formation. On the basis of this observation, we amidated the C-terminal Gly¹²⁶ of PrP106–126, seeking to decrease the fibrillogenic ability of the peptide. This in turn allowed us to assess the relationship between aggregation state and biological activity as well as the influence of the substitution Val for Ala¹¹⁷ on the physicochemical characteristics. The amidation of the C-terminus abolished the molecular polymorphism of PrP106–126 yielding a predominantly random coil structure at both neutral and acidic pH, and increased the sensitivity to the helix-promoting solvent TFE. Interestingly, although this change also decreased the propensity of the peptide to generate amyloid fibrils, the fibrillogenic ability was not completely abolished. A possible interpretation of these results is that PrP106–126 molecules assemble in an anti-parallel β -pleated sheet structure, which is stabilized by head-to-tail ion-pair interactions between Lys¹⁰⁶ and Lys¹¹⁰ and the carboxy group of Gly¹²⁶ (Figure 4). Elimination of the charge at the C-terminus shifts the equilibrium towards the random-coil monomeric state. However, the finding that PrP106–126 NH₂ still forms a limited number of fibrils suggests that van der Waals interactions involving the hydrophobic segment of the peptide contribute to fibril assembly.

Among prion-related encephalopathies, GSS disease is characterized by the highest degree of parenchymal amyloid [30]. This disorder segregates with mutations of the PrP gene. Because one of these mutations (i.e. Ala¹¹⁷ → Val) is contained in the PrP106–126 region, we studied its effects on the peptide's conformational and fibrillogenic properties. Valine is a well-known β -sheet structure promoter [31,32] that increases the hydrophobic profile of the C-terminal part of PrP106–126. By molecular dynamic

simulations of a similar peptide, PrP109–122, Kazmirski et al. [33] found that substitution of Val for Ala¹¹⁷ was the only helix-destabilizing modification of a variety of replacements with hydrophobic amino acid residues [33]. PrP106–126 V did not demonstrate a fibrillogenic ability superior to that of PrP106–126. However, the importance of this substitution was apparent by comparing PrP106–126 NH₂ and PrP106–126 VNH₂, because neither had a carboxy contribution to aggregation. The presence of Val at position 117 increased the aggregation properties of the amidated peptide; fibril morphology was very similar to that of PrP106–126.

With regard to the relationship between aggregation state and biological activity of PrP peptides, our results indicate that amyloid fibrils are not required for neurotoxicity because the effects of PrP106–126 NH₂ on primary neuronal cultures were similar to those of the wild-type sequence. Conversely, under our experimental conditions, astroglial proliferation seemed to be related to the presence of amyloid fibrils. Therefore it is possible that astrogliosis in prion encephalopathies without amyloid deposits is a mediated effect (e.g. microglia-dependent) rather than a direct effect of disease-specific PrP isoforms.

In conclusion, our results support the view that the PrP region spanning residues 106–126 is susceptible to conformational transition and might be the nido at which the conversion of PrP^C to PrP^{Sc} is initiated. Furthermore, evidence suggests that, within this region, His¹¹¹ is important to the structural flexibility as well as the stability of the newly formed β -sheet structure. PrP106–126 and PrP106–126 NH₂ can be considered to be complementary models for investigation of the pathogenesis of prion diseases. The former is suitable for short-term studies *in vitro*; however, its use is limited by its poor manageability. This is at least partly related to the free carboxy ion on Gly¹²⁶, which is moreover not present in the intact protein. Removal of this charge results in a peptide that retains neurotoxic activity and mimics the slow kinetics of fibril formation observed *in vivo* in prion-related encephalopathies.

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REFERENCES

- 1 Prusiner, S. B. (1991) *Science* **252**, 1515–1522
- 2 Bolton, D. C., McKinley, M. P. and Prusiner, S. B. (1982) *Science* **218**, 1309–1311
- 3 McKinley, M. P., Bolton, D. C. and Prusiner, S. B. (1983) *Cell* **35**, 57–62
- 4 DeArmond, S. J., McKinley, M. P., Barry, R. A., Brautfield, M. B., McColloch, J. R. and Prusiner, S. B. (1985) *Cell* **41**, 221–235
- 5 Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. and Tagliavini, F. (1993) *Nature (London)* **362**, 543–546
- 6 Forloni, G., Del Bo, R., Angeretti, N., Chiesa, R., Smiraldi, S., Doni, R., Ghibaudi, E., Salmona, M., Porro, M., Verga, L. et al. (1994) *Eur. J. Neurosci.* **6**, 1415–1422
- 7 Brown, D. R., Schmidt, B. and Kretschmar, H. A. (1996) *Nature (London)* **380**, 345–347
- 8 Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R. and Wüthrich, K. (1996) *Nature (London)* **382**, 180–182
- 9 Riek, R., Hornemann, S., Wider, G., Glockshuber, R. and Wüthrich, K. (1997) *FEBS Lett.* **413**, 282–288
- 10 Donne, D. G., Viles, J. H., Groth, D., Mehlhorn, I., James, T. L., Cohen, F. E., Prusiner, S. B., Wright, P. E. and Dyson, H. J. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13452–13457
- 11 Caughey, B. W., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F. and Caughey, W. S. (1991) *Biochemistry* **30**, 7672–7680
- 12 Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E. and Prusiner, S. B. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10962–10966
- 13 Tagliavini, F., Prelli, F., Verga, L., Giaccone, G., Sarma, R., Gorevic, P., Ghetti, B., Passerini, F., Ghiabaudi, E., Forloni, G. et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9678–9682

- 14 Selvaggini, C., De Gioia, L., Cantù, L., Ghibaudi, E., Diomede, L., Passerini, F., Forloni, G., Bugiani, O., Tagliavini, F. and Salmona, M. (1993) *Biochem. Biophys. Res. Commun.* **194**, 1380–1386
- 15 De Gioia, L., Selvaggini, C., Ghibaudi, E., Diomede, L., Bugiani, O., Forloni, G., Tagliavini, F. and Salmona, M. (1994) *J. Biol. Chem.* **269**, 7859–7862
- 16 Brown, R. D., Herms, J. and Kretschmar, H. A. (1994) *Neuroreport* **5**, 2057–2060
- 17 Brandner, S., Isenmann, S., Raeber, A., Fischer, M., Sailer, A., Kobayashi, Y., Marino, S., Weissmann, C. and Aguzzi, A. (1996) *Nature (London)* **379**, 339–343
- 18 Diomede, L., Sozzani, S., Luini, W., Algeri, M., De Gioia, L., Chiesa, R., Lievens, P. M., Bugiani, O., Forloni, G., Tagliavini, F. and Salmona, M. (1996) *Biochem. J.* **320**, 563–570
- 19 Salmona, M., Forloni, G., Diomede, L., Algeri, M., De Gioia, L., Angeretti, N., Giaccone, G., Tagliavini, F. and Bugiani, O. (1997) *Neurobiol. Dis.* **4**, 47–57
- 20 Muramoto, T., Scott, M., Cohen, F. E. and Prusiner, S. B. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15457–15462
- 21 Yang, J. T., Wu, C. S. and Martinez, H. M. (1986) *Methods Enzymol.* **130**, 208–269
- 22 Andreoni, G., Angeretti, N., Lucca, E. and Forloni, G. (1997) *Exp. Neurol.* **148**, 281–287
- 23 Yao, J., Keri, J. E., Taffs, R. E. and Colton, C. A. (1992) *Brain Res.* **591**, 88–93
- 24 Chiesa, R., Angeretti, N., Lucca, E., Salmona, M., Tagliavini, F., Bugiani, O. and Forloni, G. (1996) *Eur. J. Neurosci.* **8**, 589–597
- 25 Jackson, G. S., Hosszu, L. L. P., Power, A., Hill, A. F., Kenney, J., Saibil, H., Craven, C. J., Walther, J. P., Clarke, A. R. and Collinge, J. (1999) *Science* **283**, 1935–1937
- 26 Chen, S. G., Teplow, D. B., Parchi, P., Teller, J. K., Gambetti, P. and Aulizio-Gambetti, L. (1995) *J. Biol. Chem.* **270**, 19173–19180
- 27 Hegde, R. S., Mastrianni, J. A., Scott, M. R., DeFea, K. A., Tremblay, P., Torchia, M., DeArmond, S. J., Prusiner, S. B. and Lingappa, V. R. (1998) *Science* **279**, 827–834
- 28 Terzi, E., Holzemann, G. and Seelig, J. (1994) *Biochemistry* **33**, 1345–1350
- 29 Terzi, E., Holzemann, G. and Seelig, J. (1994) *Biochemistry* **33**, 7434–7441
- 30 Ghetti, B., Piccardo, P., Frangione, B., Bugiani, O., Giaccone, G., Young, K., Prelli, F., Forlow, M. R., Dlouhy, S. R. and Tagliavini, F. (1996) *Brain Pathol.* **6**, 127–145
- 31 Branden, C. and Tooze, J. (1991) in *Introduction to Protein Structure* (Branden, C. and Tooze, J., eds.), pp. 247–267, Garland Publishing, New York
- 32 Warwicker, J. and Gane, P. J. (1996) *Biochem. Biophys. Res. Commun.* **226**, 777–782
- 33 Kazmirski, S. L., Alonso, D. O., Cohen, F. E., Prusiner, S. B. and Daggett, V. (1995) *Chem. Biol.* **2**, 305–315

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